FUSARIUM GRAMINEARUM SPECIES COMPLEX (FGSC) COMPOSITION IN SOUTH AFRICAN WHEAT AND MAIZE GROWN IN ROTATION

by

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SUMMARY

Fusarium graminearum is an important pathogen of economically important cereal crops. Recently, the morpho-species has been reclassified as a species complex consisting of 16 phylogenetic species, called the *Fusarium graminearum* species complex (FGSC). FGSC species cause a number of important plant diseases, including Fusarium head blight (FHB) of wheat and Gibberella ear rot (GER) of maize. Infection by these pathogens results in poor grain yield and quality and contaminates grain with noxious secondary metabolites, called mycotoxins. FGSC species produce Type B-type trichothecenes; such as nivalenol (NIV), fusarenon-X (FX), deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-ADON) and 3-acetyl-deoxynivalenol (3-ADON); and zearalenone (ZEA). The consumption of mycotoxin-contaminated grain has been associated with serious human and animal health risks.

The diversity and distribution of FGSC species have been evaluated globally, but only a single study has been conducted in South Africa. To date, six of the 16 phylogenetic species have been documented on wheat, maize and barley produced in the country. Wheat and maize, the two most important cereals grown in South Africa, are commonly rotated with each other. This is of particular concern to disease development and mycotoxin contamination of the two crops. While FGSC species have been identified on wheat and maize in South Africa, these crops were not grown in a rotational system. The aim of this study, therefore, was to investigate FGSC species composition on wheat and maize grown in a rotational cropping system in South Africa.

Identification of *Fusarium* species based exclusively on morphological characteristics has proven to be inadequate for the effective differentiation between closely related species. Therefore, the first objectives of the study was to evaluate matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS), PCR – restriction fragment length polymorphism (PCR-RFLP) and species-specific PCR for the rapid and reliable identification of FGSC species previously reported in South Africa. Different protein mass spectra obtained with MALDI-TOF MS were able to separate the FGSC species reported in South Africa. Double restriction digestion of the *translation elongation factor α-1* (*EF*-1α) gene region of local FGSC species with *Bfa*I and *Bsa*HI was able to distinguished *F. graminearum* s.s., *F. cortaderiae* and *F. acaciae-mearnsii* from each other and from the remaining two FGSC species in a single restriction digest. The restriction profile produced by *Ear*I, when the *histone* (*H*3) gene region was digested, distinguished *F. boothii* from the FGSC species evaluated. These techniques, thus, could be used to identify FGSC species present on cereal crops in South Africa.

The second objective of the study was to identify FGSC species on wheat and maize grown in rotation and their chemotypes. The *in vitro* production of nivalenol (NIV), fusarenon-X FX, deoxynivalenol DON and 15-acetyl-deoxynivaleno 15-ADON was also determined. The most isolated FGSC species from wheat and maize was *F. graminearum* s.s. Only one isolate from wheat was identified as *F. boothii.* No other FGSC species were isolated from maize, but several non-FGSC species were associated with diseased maize ears. 15-ADON was the predominant chemotype of the FGSC identified in South Africa. Seven isolates produced the NIV chemotype, but none of these isolates were FGSC species. Cultural practices, such as crop rotating with hosts of the FGSC species, needs to be further evaluated as crop rotation in combination with other variables potentially favoured the occurrence of *F. graminearum* s.s.

OPSOMMING

Fusarium graminearum is 'n belangrike patogeen van ekonomies belangrike graan gewasse. Die morfo-spesie is onlangs geherklassifiseer as 'n spesie kompleks wat bestaan uit 16 filogenetiese spesies, naamlik die *Fusarium graminearum* spesie kompleks (FGSK). FGSK spesies veroorsaak belangrike graan siektes, insluitend Fusarium aarskroei van koring en Gibberella kopvrot van mielies. Infeksie deur hierdie patogene veroorsaak swak graan opbrengte en kwaliteit, en besoedel graan met giftige sekondêre metaboliete wat bekend staan as mikotoksiene. FGSK spesies produseer tipe-B trichothecenes (TCT-B); veral nivalenol (NIV), fusarenon-X (FX), deoxynivalenol (DON), 15-asetiel-deoxynivalenol (15-ADON) en 3-asetiel-deoxynivalenol (3-ADON); en zearalenone (ZEA). Die inname van mikotoksien-besmette graan gaan gepaard met ernstige menslike en diere gesondheid risiko's.

Die diversiteit en verspreiding van die FGSK is al wêreldwyd geëvalueer, maar daar is slegs 'n enkele studie in Suid-Afrika gedoen. Tot op hede is ses van die 16 filogenetiese spesies gedokumenteer op koring, mielies en gars in Suid Afrika. Koring en mielies is die twee belangrikste verboude grane in Suid-Afrika en word algemeen geroteer in wisselbou. Wisselbou is van kritiese belang vir siekte ontwikkeling en mikotoksien besmetting van hierdie twee gewasse. Die koring en mielies in Suid-Afrika, waarvandaan die FGSK spesies geïdentifiseer is, was nie geroteer met mekaar nie. Die doel van hierdie studie was dus om die FGSK spesiesamestelling op koring en mielies, wat saam in wisselbou stelsels in Suid-Afrika gebruik word, te ondersoek.

Die identifisering van *Fusarium* spesies was uitsluitlik gebaseer op morfologiese kenmerke, maar was onvoldoende vir die effektiewe onderskeid tussen naverwante spesies. Die eerste doelwit van hierdie studie was dus om “matrix-assisted laser desorption ionization–time of flight mass spectrometry” (MALDI-TOF MS), Polimerasie Ketting Reaksie beperkings fragment lengte polimorfisme (PKR-RFLP) en spesie-spesifieke PKR te evalueer as vinnige en betroubare identifikasie tegnieke vir die FGSK spesies, wat voorheen in Suid-Afrika berig is. Verskillende proteïn massa spektra was verkry met MALDI-TOF MS en was in staat om die FGSK spesies te onderskei. Dubbel restriksie ensiem vertering met *Bfa*I en *Bsa*HI kon *F. graminearum* s.s., *F. cortaderiae* en *F. acaciae-mearnsii* van mekaar onderskei, terwyl die oorblywende twee FGSK spesies met ’n verdere enkel restriksie ensiem vertering onderskei kon word. Die profiel wat geproduseer is met *Earl*, waar die *histoon* (*H3*) geen verteer is, onderskei *F. boothii* van die FGSK spesies wat geëvalueer is. Hierdie tegnieke kan dus gebruik word om FGSK spesies in Suid-Afrikaanse graangewasse te identifiseer.

Die tweede doelwit van hierdie studie was om FGSK spesies en hul chemotipes, wat voorkom op koring en mielies wat in wisselbou gebruik word met mekaar, te identifiseer. Die *in vitro* produksie van NIV, FX, DON en 15-ADON is ook bepaal. Die mees geïsoleerde FGSK spesies van koring en mielies was *F. graminearum* s.s. Slegs een isolaat van koring is geïdentifiseer as *F. boothii*. Geen ander FGSK spesies was geïsoleer vanaf mielies nie, maar verskeie nie-FGSK spesies was geassosieer met besmette mielies. 15-ADON was die oorheersende chemotipe van die FGSK spesies wat geïdentifiseer is in Suid-Afrika. Sewe isolate produseer die NIV chemotipe, maar nie een van hierdie isolate is FGSK spesies nie. Kulturele praktyke, soos wisselbou met gashere van die FGSK spesies, moet verder geëvalueer word siende dat rotasie met graan gewasse die voorkoms van *F. graminearum* s.s bevorder het.

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CONTENTS

CHAPTER 1

The *Fusarium graminearum* species complex associated with wheat and maize in South Africa

INTRODUCTION

Wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) are amongst the most important food commodities worldwide (Byerlee and Eicher, 1997). These cereal crops constitute major staple foods for many Africans, with maize being consumed by approximately 200 million people in developing African countries (Shephard *et al*., 2007; Shephard, 2008). Furthermore, these cereals are also extensively used for animal feed. The production of wheat and maize is threatened by many fungal pathogens, of which *Fusarium graminearum* Schwabe is amongst the most important (Kazan *et al*., 2012). The infection of cereal grains by *F. graminearum* causes numerous diseases which include Fusarium head blight (FHB) of wheat, barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) (Marasas *et al*., 1988; Scott *et al*., 1988). The fungus also causes Gibberella ear rot (GER) (Marasas *et al*., 1979) and Gibberella stalk rot of maize (Moreno-Gonzalez *et al*., 2004)

The *F. graminearum* species was previously considered a single morphological species. Recent molecular studies, however, have indicated that it constitutes a species complex known as the *Fusarium graminearum* species complex (FGSC) (O'Donnell *et al*., 2000; 2004; 2008; Starkey *et al*. 2007; Sarver *et al*., 2011). The FGSC consists of 16 phylogenetically distinct species (Fig. 1), also referred to as *F. graminearum* sensu lato (s.l.). Six of these species have been reported on South African grains; namely *F. graminearum* sensu stricto (s.s.) Schwabe*, F. cortaderiae* O'Donnell, T. Aoki, Kistler *et* Geiser*, F. acaciae-mearnsii* O'Donnell, Aoki, Kistler *et* Geiser*, F. meridionale* Aoki, Kistler, Geiser *et* O'Donnell, *F. boothii* O'Donnell, Aoki, Kistler *et* Geiser and *F. brasilicum* Aoki, Kistler, Geiser *et* O'Donnell.

Grains contaminated with FGSC species are often contaminated with mycotoxins, which can have serious health implications for both humans and animals (Windels, 2000; Bennett and Klich, 2003, Suga *et al*., 2008). Type B trichothecenes (TCT-B) and zearalenone (ZEA) are two of the major mycotoxin groups produced by species within the FGSC. The occurrence of FGSC species and their associated mycotoxins on economically important grain crops has resulted in the regulation of these mycotoxins in more than 100 countries (Van Egmond, 2002; Barug *et al*., 2003; Fellinger, 2006). The European Scientific

Committee for Food has suggested limits for TCT-B, whereby temporary tolerable daily intake of nivalenol (NIV) is 0.7 µg kg^{-1} body weight and for deoxynivalenol (DON) is 1 µg kg^{-1} body weight (Schothorst and Van Egmond 2004). The advisory levels recommended for the Unite States of America by the Food and Drug Administration (FDA) for DON is 1 ppm on finished wheat products consumed by humans (Food and Drug Administration, 2010). However, South Africa has no legislation with respect to maximum levels of *Fusarium*-associated mycotoxins on food or feed (Van Egmond, 1993).

Fusarium graminearum does not only contaminate cereal crops with mycotoxins. It also causes reductions in crop yield and grain quality. Poor grain quality, characterised by grain damage, low grain density and the presence of disease symptoms, is down-graded and in turn negatively impacts the grain industry (Windels, 2000). The primary production and agro-processing sectors of South Africa accounts for about 14% of the country's GDP (Department of Agriculture, Forestry and Fisheries, 2012a), demonstrating the importance of the agricultural sector to the South African economy. Thus, this review will focus on the importance of wheat and maize in South Africa and the importance of FGSC species on their production.

PRODUCTION OF WHEAT AND MAIZE IN SOUTH AFRICA

Importance of wheat and maize

Wheat and maize are the most important cereal crops produced in South Africa (Department of Agriculture, 2003). The country produces approximately 1.5 to 3 million tons of wheat annually, and it has been estimated by the United States Department of Agriculture Foreign Agricultural Services (2015) that approximately 13 million tons of maize will be produced during the 2015/16 marketing year. Wheat is produced in all nine provinces of South Africa (Van Niekerk, 2001), with the Western Cape and Free State being the major wheat-producing provinces (Department of Agriculture, Forestry and Fisheries, 2012b). In the winter rainfall regions wheat is mostly planted between April and June, and in the summer rainfall regions it is predominantly planted between May and July (Department of Agriculture, Forestry and Fisheries, 2010). The Free State, North West and Mpumalanga provinces are the main maize-producing regions of South Africa (South African Grain Laboratory, 2011). Maize is planted between October and December in summer rainfall areas where rainfall exceeds 350 mm per annum (Department of Agriculture, 2003).

Wheat is largely utilised as food for humans globally (Department of Agriculture, Forestry and Fisheries, 2010). Finely milled wheat, known as flour, is used in the bread and baking industry. South Africa primarily produces wheat for human consumption, but a small percentage of wheat is used as feed for animals (Department of Agriculture, Forestry and

Fisheries, 2010). Southern and eastern parts of Africa maize is considered the most important staple food (Byerlee and Eicher, 1997). Consumption levels as high as 400 to 500 g of maize per person per day have been reported in rural areas of Africa (Shephard, 2008). White maize is predominately used for human consumption, while yellow maize is utilized as animal feed (Department of Agriculture, Forestry and Fisheries, 2012b).

Fusarium species on wheat and maize

Cereal production can be greatly limited by phytopathogens that cause disease that reduce grain yield and quality. Wheat diseases are caused by a variety of bacterial, viral and fungal pathogens of which the fungal genus *Fusarium* is associated with numerous diseases (Gorlach *et al*., 1996). FHB, as well as root and crown rot of wheat, have been associated with a number of *Fusarium* species including the FGSC species, *F. avenaceum* Wollenweber & Reinking, *F. brachygibbosum* Padwick, *F. cerealis* (Cooke) Sacc., *F. chlamydosporum* Wollenweber & Reinking, *F. culmorum* (W.G. Smith), *F. incarnatum-equiseti* (syn. *F. equiseti* (Corda) Saccardo), *F. lunulosporum* Gerlach, *F. oxysporum* Schlechtendahl emend. Snyder & Hansen, *F. poae* (Peck) Wollenweber, *F. pseudograminearum* Aoki & O'Donnell, *F. solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen and *F. tricinctum* (Corda) Saccardo (Parry *et al.*, 1995; Ruckenbauer *et al.*, 2001; Boutigny *et al*., 2011; van Coller *et al*., 2013; Beukes, 2015). The predominant *Fusarium* phytopathogens of maize are the FGSC species, *F. verticillioides* (Sacc.) Nirenberg*, F. proliferatum* (Matsushima) Nirenberg and *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (Munkvold, 2003b; Boutigny *et al*., 2012). Wheat and maize are often rotated with each other in the Limpopo, Northern Cape and KwaZulu-Natal provinces to limit soil erosion, increase fertility and generate additional income from the subsequent crop production (Mr. A. Pistorius, personal communication). The rotation of two or more susceptible hosts and improper sanitation practices, however, may increase disease and/or mycotoxin contamination in farmer fields.

THE *FUSARIUM GRAMINEARUM* SPECIES COMPLEX

The fungal genus *Fusarium* belongs to the phylum *Ascomycota*, class *Ascomycetes* and order *Hypocreales* (Leslie, 1995), and consists of species that are ubiquitous in nature (Nelson *et al*., 1983; Logrieco *et al*., 2003). Its classification, however, is extremely complex due to insufficient morphological differences, large host range and the clonal nature of many species. Species within the *Fusarium* genus are humicolous and facultative saprophytes as they occupy soil and are found on living and dead organic material (Pomeranz *et al.,* 1990).

The genus *Fusarium* was first described by Link (1809). It was, however, Wollenweber (1931) that recognised the distinctiveness of several species, and divided them into 16 sections, 65 species, and 77 sub-specific varieties and forms. Booth (1971) made an important contribution to *Fusarium* taxonomy when the morphological characteristics of conidiogenous cells were introduced to distinguish between closely-related *Fusarium* species. To date, Booth's method is still used to differentiate between species in the *Liseola* and *Sporotrichiella* sections (Leslie and Summerell, 2006). After the 1990's, genetic and molecular techniques became popular in defining Fusarium species (Ouelett and Seifert, 1993). Morphological species were thus separated in several 'biological' and 'phylogenetic' species. The "One Fungus, One Name" concept was proposed in 2013 to support good taxonomic practices within *Fusarium* in accordance to changes in the International Code of Nomenclature for algae, fungi and plants (Geiser *et al*., 2013).

Fusarium graminearum is one of the most widely studied and important *Fusarium* species in the world. It only produces macroconidia, which range between 2.5–5 x 35–62 µm and consist of three to seven septates (Cappellini and Peterson, 1965; Booth, 1971; Sutton, 1982). The species was divided into several new species, collectively known as the FGSC, by O'Donnell *et al*. (2000) using multi-locus genotyping (MLGT), combined with genealogical concordance of phylogenetic species recognition (GCPSR) (Taylor *et al*., 2000; Starkey *et al*., 2007; Aoki *et al*., 2012). The FGSC comprises 16 phylogenetically distinct species, including *F. graminearum* s.s*., F. cortaderiae, F. acaciae-mearnsii*, *F. meridionale*, *F. boothii*, *F. brasilicum*, *F. asiaticum* O'Donnell, Aoki, Kistler *et* Geiser, *F. austroamericanum* Aoki, Kistler, Geiser *et* O'Donnell, *F. mesoamericanum* Aoki, Kistler, Geiser *et* O'Donnell, *F. gerlachii* Aoki, Starkey, Gale, Kistler & O'Donnell, *F. vorosii* Toth, Varga, Starkey, O'Donnell, Suga & T. Aoki, *F. aethiopicum* O'Donnell, Aberra, Kistler *et* Aoki*, F. ussurianum* Aoki, Gagkaeva, Yli-Mattila, Kistler, O'Donnell, *F. nepalense* Aoki, Carter, Nicholson, Kistler & O'Donnell, *F. louisianense* Gale, Kistler, O'Donnell & Aoki and *Fusarium* species (Fig. 1) (O'Donnell *et al*., 2000, 2004, 2008; Starkey *et al*. 2007; Yli-Mattila *et al*., 2009).

The FGSC is believed to have originated in the southern hemisphere (O'Donnell *et al.*, 2004; Starkey *et al*., 2007) and Asia (Yli-Mattila *et al*., 2009). Recent surveys, however, have reported the distribution of FGSC species in the United States, Canada and Europe (O' Donnell *et al*., 2000, 2004; Láday *et al*., 2004; Ramirez *et al*., 2007; Lee *et al*., 2009; Desjardins *et al*., 2011). This global distribution of the FGSC species is attributed to the import and export of cereal commodities as well as the fluctuating environmental and climatic conditions (Qu *et al*., 2008). Geographic location and the type of host also have been proposed to affect FGSC species distribution (O Donnell *et al*., 2000; Lee *et al*., 2009).

Species of the FGSC have been reported on different crops hosts which include maize, wheat, barley, rice and other cereal crops (Van der Lee *et al*., 2015). Numerous surveys have been completed on the FGSC species on wheat, but limited studies were conducted on maize (Boutigny *et al*., 2011). *Fusarium graminearum* s.s. is the predominant FGSC species found on wheat worldwide, and severely affects several other agriculturally important crops, including barley and maize. The species *F. boothii* and *F. meridionale* is commonly found on maize *(*Van der Lee *et al.,* 2015), whereas *F. asiaticum* is commonly found on rice (Zhang *et al*., 2012). A study in South Africa identified *F. graminearum* s.s. as the primary FGSC species found on wheat, while *F. boothii* was exclusively associated with maize ears (Boutigny *et al*., 2011).

ETIOLOGY OF THE *FUSARIUM GRAMINEARUM* SPECIES COMPLEX

Understanding the life cycle and epidemiology of FGSC species is important for the management of diseases on cereal crops (Zeller *et al*., 2003). Disease severity varies from season to season, and depends on the presence of primary inoculum and favourable environmental conditions from flowering until kernel development (McMullen and Stack, 1999). Planting of host plant, such as wheat, maize and sorghum in the same production areas and fields may serve as a reservoir of inoculum for the subsequent planting of susceptible crops (Guo *et al*., 2008).

Production and dispersal of inoculum

Environmental factors play an important role in the production and dispersal of FGSC species. The formation, maturation and release of macroconidia (asexual spores) and ascospores (sexual spores) is favoured by maximum daily temperatures ranging between 24°C and 28°C, light rainfall and irrigation (Booth, 1971; Reid *et al*., 1999). Ascospores, which are produced in perithecia, established on debris from the previous crop found on soil surfaces (Cook, 1981; Jones and Clifford, 1983), are predominately discharged at night (Munkvold, 2003b), when relative humidity increases. Heavy rainfall, however, inhibits the release of ascospores (Paulitz, 1996). Wind, rain and insects play an important role in the dispersal of macroconidia (Stutton, 1982; Parry *et al*., 1995). Airborne macroconidia and ascospores are deposited onto the florets of wheat (Fig. 2) and silks of maize (Fig. 3), from where infection is initiated. Insects deposit fungal spores onto susceptible host tissue while feeding on maize silks and husks, which can result in kernel infection (Munkvold, 2003b).

Infection and colonisation

Infection and colonisation of crops by FGSC occur after the primary inoculum (infectious propagules) comes in contact with susceptible host tissue. In wheat, spikelets are infected mostly during anthesis (Stutton, 1982). After the primary inoculum has germinated, the fungal hyphae grows on the exterior surfaces of the florets and glumes, and then enters the host via natural openings such as stomata (Bushnell *et al*., 2003). It invades the host via subcuticular hyphae and bulbous infection hyphae (Rittenour and Harris, 2010). The pathogen spreads between wheat florets and spikelets through the vascular bundles. Colonization of the xylem and phloem results in the dysfunction of the vascular tissue, which causes premature death of the spikelet (Goswami and Kistler, 2005).

Infection of maize by the FGSC mainly occurs during early silking and pollination (Viger *et al*., 2001). The silks serve as the primary pathway for infection of maize kernels. Silks younger than 6 days are highly susceptible and easily infected, but their susceptibility decreases as the silks ages (Enerson and Hunter, 1980). Wounds created by birds and insects also provide a point of entry for the pathogen (Munkvold, 2003b). Kernel moisture during silking, as well as favourable environmental condition; such as mild temperatures and high rainfall; favours the development of GER on maize (Sutton, 1982).

Disease symptoms

Wheat heads affected by FHB have a bleached and tan appearance due to the loss of chlorophyll that might affect single or groups of spikelets (Fig. 4) (Parry *et al*., 1995; McMullen and Stack, 1999). During favourable climatic conditions, pink to salmon orange fungal growth may be observed on the infected spikelet, glumes and kernels (Steffenson, 2003). Kernels become shrunken, withered and light, and are reduced in number (McMullen *et al*., 1997). The severity of the kernel symptom depends on the time of infection (Goswami and Kistler, 2005).

Symptoms caused by FGSC on maize first become visible as white fungal mycelia at the apex of the maize ear which, over time, turns dark pink (Fig. 5) (Goswami and Kistler, 2005). The fungal mycelium then progresses down towards the base of the maize ear (Goswami and Kistler, 2005). If an infection occurs at an earlier stage the whole maize ear may rot and will be covered by pink mycelia. This causes the husk to adhere tightly to the maize ear (White, 1999). Infected maize kernels are often small in size, shrivelled and broken. Fusarium head blight and GER symptomatic grain result in poor grain quality and inevitably price reductions due to reduced grading. In addition, their grains may be highly contaminated with mycotoxins.

MYCOTOXINS ASSOCIATED WITH THE *FUSARIUM GRAMINEARUM* SPECIES COMPLEX

Mycotoxins are secondary metabolites produced by fungi in food and feed (Zinedine and Mañes, 2009). The most important fungi that produce mycotoxins include species of the genera *Aspergillus*, *Penicillin* and *Fusarium* (Van der Lee *et al*., 2015). Infection of crops with mycotoxigenic fungi often takes place in the field, while toxin production occurs both in the field and during storage. Improper storage, shipping and handling of infected food and feed may aggravate mycotoxin contamination if conditions favour fungal growth and mycotoxin production.

Species of the FGSC produce two important groups of mycotoxins: the TCT-Bs and ZEA (D'Mello and Macdonald, 1997). The TCT-Bs include DON, commonly known as vomitoxin, and its derivatives 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON). They also include NIV and its derivative is 4-acetyl nivalenol also known as fusarenon-X (FX) (Ward *et al*., 2002; Goswani and Kistler, 2005; Starkey *et al.*, 2007; O'Donnell *et al.*, 2008). The TCT-Bs consist of a 12, 13-epoxytrichothene skeleton and an olefinic bond with various side chain substitutions (Bennett and Klich, 2003), while ZEA is a nonsteroidal molecule with estrogenic properties (Kuiper-Goodman *et al*., 1987; Boutigny *et al*., 2012). DON is the most dominant TCT-B produced by the FGSC and is often found in wheat, maize and barley (Zinedine and Mañes, 2009).

Mycotoxin production

Genes associated with the TCT biosynthesis are clustered within the genomes of FGSC species. FGSC species that produce NIV possesses a functional *Tri*13 and *Tri*7 gene that are not present in DON producers (Brown *et al*., 2001; Lee *et al*., 2002; Ji *et al*., 2007). The type of TCT produced by FGSC isolates can be determined with primers that distinguish the 15-ADON, 3-ADON and NIV chemotypes (Ward *et al*., 2002; 2008; Nicholson *et al*., 2004). Acetylation of TCTs alters their biological activity and toxicity (Kimura *et al*., 1998), which have important health consequences (Kimura *et al*., 1998). DON is considered to be less toxic than the other TCTs (Zinedine and Mañes, 2009).

Importance of mycotoxins

FGSC mycotoxins have been associated with health problems in humans and animals. Immuno-suppression and neurological disorders have also been associated with the consumption of TCT-B-contaminated grain (Bennett and Klich, 2003). DON is a strong inhibitor of eukaryote protein biosynthesis (Goswami and Kistler., 2004). Swine poisoned by DON-contaminated feed displayed symptoms such as vomiting, suppressed immune

function, diarrhoea, alimentary haemorrhaging and feed refusal (McMullen *et al*., 1997; Bennett and Klich, 2003). NIV is more toxic than DON to both humans and domestic animals (Ryu *et al*., 1988; Schothorst and Van Egmond, 2004). ZEA has estrogenic properties that cause reproductive problems in animals that include abortion in swine, reduced litter size and male infertility (Nelson *et al*., 1993). ZEA also has the potential to stimulate breast cancer cells, which may result in health consequences for humans (Ahamed *et al*., 2001; Yu *et al*., 2005).

Regulation of mycotoxins produced by FGSC species

Food commodities contaminated with mycotoxins have been classified as a major dietary risk factor. The risk of mycotoxin contamination has been ranked more important than food additives, pesticide residues or synthetic contaminates (Zinedine and Mañes, 2009). The presence of mycotoxins, therefore, is regulated in over 100 countries worldwide (Haumann, 1995; Van Egmond *et al*., 2007). No regulations governing the maximum allowable levels of any of the *Fusarium* related mycotoxins in food and feed currently exists in South Africa.

The European commission has set limits for mycotoxin contamination of food commodities depending on the level of processing. Unprocessed cereal grains, such as wheat and maize, should not contain DON exceeding 1250 ppb (European Food Safety Authority, 2014). Grain used in the preparation of bread, breakfast cereals and pastries should not be contaminated with more than 500 ppb of DON (European Food Safety Authority, 2014). The European commission has also set limits for ZEA contamination of cereals. Unprocessed cereals such as wheat should not exceed more than 100 ppb, while ZEA in cereals intended for human consumption in the form of flour or bran should not exceed 75 ppb (European Food Safety Authority, 2014). Cereals used in the making of bread, breakfast cereals and pastries should not be contaminated with ZEA of more than 50 ppb (European Food Safety Authority, 2014).

In the USA, the limit for DON in finished wheat products destined for human consumption was set at 1 ppm (U.S. Food and Drug Administration, 2010). DON in wheat grain and grain by-products, such as bran, was set at 10 ppm (U.S. Food and Drug Administration, 2010). Wheat grains subjected to distilling and brewing processes were limited to 30 ppm (U.S. Food and Drug Administration, 2010). Limits were also set for animal feed according to the type of animal. According to the FDA, swine feed should not contain more than 5 ppm of DON (U.S. Food and Drug Administration, 2010) and cattle feed should not exceed 10 ppm DON (U.S. Food and Drug Administration, 2010).

DISEASE CONTROL OF MYCOTOXIGENIC *FUSARIUM* SPECIES

The management of FGSC species to reduce yield losses and mycotoxin contamination of cereal crops can be divided into pre-and postharvest strategies.

Pre-harvest strategies

Cultural management: Crop cultivation practises can significantly impact disease and mycotoxin contamination by FGSC species. Fields with no history of the pathogen should be selected as it would potentially limit the inoculum present when host crops are planted (Tekauz *et al*., 2000). Crop rotation with non-host crops such as legumes and brassicas has been suggested as an effective cultural practice for managing FGSC inoculum in grain fields (Munkvold, 2003a; Yli-Mattila *et al*., 2009). In fields previously cultivated with maize or wheat, seed beds should be prepared that will reduce inoculum build-up if practical (Munkvold, 2003a). Volunteer plants which serve as over-wintering sources for the pathogen should be eradicated by removal, early ploughing and burning (Munkvold, 2003b). Practises such as deep ploughing can also be used to bury crop debris on the soil surface (Krebs *et al*., 2000; Blandino *et al*., 2010). This is especially important when fields are replanted to crops affected by the same *Fusarium* species, such as maize, wheat and sorghum (Beukes, 2015). The disadvantages of cultural practices are that ploughing and burning often result in erosion and the loss of soil moisture, which is essential for crop growth (Steffenson, 2003). Cultural practices that reduce plant stress are essential for reducing diseases of crops (Nel, 2005). Soil moisture should also be adequately controlled for optimal plant growth (Mukanga *et al.,* 2011). Recommended plant densities and row widths should be complied with to reduce water stress (Mukanga *et al.,* 2011). Crops should be fertilized with the correct concentrations of nitrogen and other essential plant nutrients present in soil (Blandino *et al*., 2008). Pre-harvest herbicides can be applied to control weeds and alternative host plants that may compete with crops for nutrients, water and space (Jones *et al*., 1980). The weeds paradoxa grass (*Phalaris paradoxa* L.) and wild oats (*Avena fatua* L.) are both hosts of *F. graminearum* (Atanasoff, 1920; Jenkinson and Parry, 1994), and should be removed from wheat fields to prevent FHB (Atanasoff, 1920; Jenkinson and Parry, 1994). In addition to cultural practices, Good Agricultural Practices (GAP) is an important factor which should be considered (FAO, 2001; 2002). Good agricultural practices serves as a tool for the appropriate management of all sectors of primary food production. Additionally GAP is a complimentary approach to the Hazard Analysis and Critical Control Point (HACCP). Hazard Analysis and Critical Control Point is intended to prevent safety problems such as food contamination by mycotoxins (Magan and Olsen, 2004).

Chemical control: For the control of FHB of wheat, fungicide application during anthesis serves as an adequate control measure (Yin *et al*., 2009). The fungicides most effective for

the control of FHB include tebuconazole and prochloraz (Parry *et al.*, 1995). Other triazole-based fungicides, such as metconazole and prothioconazole, also reduce FHB and DON (McMullen *et al*., 2000; Hershman *et al*., 2004; Paul *et al*., 2005). None of these fungicides, however, are registered for the control of FHB of wheat in South Africa (Kriel and Pretorius, 2005). In the United Kingdom, Europe and the USA, disease forecasting models are often used to set up effective spraying schedules (Moschini and Fortugno, 1996; Xu, 2003). Fungicides are not able to control maize ear rot diseases and prevent mycotoxin accumulation in maize grain (Munkvold, 2003a). This is most likely due to husks that prevent them from making contact with the fungus. To date, no fungicides have been registered in South Africa for the control of maize ear rot pathogens (Janse Van Rensburg, 2012).

*Biological control***:** Biological control of plant pathogens has become popular due to stricter regulations with regard to pesticide (Bale *et al*., 2008). Biological control can be defined as the use of an organism to reduce the population density of another organism, or by the reduction of pests and pest effects through the use of natural enemies (Bale *et al*., 2008). *Bacillus*, *Cryptococcus* and *Trichoderma* species have all reduced the incidence of FHB in wheat and barley (Kahn *et al*., 1998; Luo and Bleakley, 1999; Gilbert and Tekauz, 2000). FHB was controlled and DON contamination reduced when non-pathogenic antagonists such as *Phoma betae* A.B. Frank and *Trichoderma* species were evaluated under greenhouse conditions (Siva and Chet, 1986; Schisler *et al*., 2002; Diamond and Cooke, 2003; Musyimi *et al*., 2012). Biological control methods for FHB are often used in combination with chemical control methods (Da Luz *et al*., 2003). It has been reported that the chemicals such as tannic acid, Chinese galls (*Galla chinensis*) and dried bark from buckthorn (*Frangula alnus*), reduces FHB disease and mycotoxin content (Forrer *et al*., 2014).

*Resistance***:** Plant resistance is considered an economically viable and environmentally sound management strategy to control FHB and GER. Host resistance in grain crops can be improved by both conventional breeding and genetic engineering (Munkvold and Desjardins, 1997; Iken and Amusa; 2004). Some wheat cultivars were bred to mature early, as they are then less susceptible to FHB than cultivars that mature later (Schroeder and Christensen, 1963). The wheat variety Sumai 3 is commonly been used as a resistant source against FHB globally (Rudd *et al*., 2001). The most important quantitative trait loci (QTLs) for FHB resistance are located on the short arm of the Sumai chromosome 3B (Anderson *et al*., 2001). The environment does not influence the stability of the Sumai 3 variety as compared to other sources of FHB resistance (Rudd *et al*., 2001). Maize inbred lines with increased resistance to GER have also been released (Reid *et al*., 2001a; b; 2003). Progress, however, has been slow to produce GER-resistant cultivars due to the quantitative nature of

resistance. The mechanism of resistance to GER remains unclear, but changes in phenolics and phenylpropanoids in silks have been linked to reduced GER and mycotoxin contamination (Assabgui *et al*., 1993; Miller *et al*., 1997; Cao *et al*., 2011).

Post-harvest strategies

Post-harvest management of grain is important to limit the growth of mycotoxigenic *Fusarium* species. The moisture content of grain at harvest can contribute to fungal proliferation and mycotoxin contamination, and should thus be reduced (Magan and Olsen, 2004). Contamination of grain with DON does not increase significantly during storage when the moisture level at harvest is <14%, and when optimal moisture, temperature and pests are controlled during storage (Richard, 2007). The separation of visually-infected grain from apparently healthy grain can significantly reduce mycotoxin contamination. Abbas *et al*. (1985) reported a reduction in DON content of 6-19% when symptomatic wheat kernels were removed. In the former Transkei region of South Africa, a 71% reduction in fumonisins was achieved when subsistence farmers sorted mouldy maize kernels from healthy ones (van der Westhuizen *et al*., 2011). The washing of maize kernels with distilled water resulted in a 65% DON and 61% ZEA reduction, while the use of a 1 M sodium carbonate solution further resulted in reductions of DON and ZEA (Trenholm *et al*., 1992). Harsh pH conditions (pH>12) for 2 days at 80^{o}C is required for the effective breakdown of DON, NIV and ZEA (Lauren and Smith, 2001). The chemical detoxification (ozone treatment) of DON has been demonstrated (Young *et al*., 2006), while ZEA by-products were undetectable following its degradation by ozone gas (McKenzie *et al*., 1997).

CONCLUSION

The infection of economically important cereal crops by FGSC species and the mycotoxin contamination of grains pose a serious food security and food safety concern to the majority of South Africans. Maize and wheat are the main staple food crops grown in South Africa. FHB of wheat and GER of maize, both caused by FGSC species, are affecting these crops, thereby posing a challenge to both commercial and subsistence farmers in the country. A proper understanding of the diversity and distribution of FGSC species in South Africa is important to manage FER and GER pathogens. Therefore, accurate pathogen identification, knowledge on host specificity and/or host preference, as well as information on production practices are required.

Insufficient morphological characteristics complicated the identification of closely-related FGSC species. Molecular techniques, in contrast, have provided a means to rapidly and accurately identify FGSC species. The first aim of this study, therefore, was to develop

identification techniques for the rapid and accurate identification of five FGSC species previously reported in South Africa. Molecular techniques that were investigated in **Chapter 2** include matrix assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS), PCR–restriction fragment length polymorphism (PCR-RFLP) and the use of a *F. graminearum*-specific primer set. MALDI-TOF MS has been reported to be an easy method for the identification of bacteria and yeast (Dong *et al*., 2009; Sitterle *et al*., 2014; Patel, 2015). Matrix assisted laser desorption ionization – time of flight mass spectrometry has also been used for the identification of clincaly important filamentous fungi (Becker *et al*., 2014; Levesque et *al*., 2015) and has furthermore been used for the identification of *Fusarium* and *Trichoderma* species (Kemptner *et al*., 2009; Dong *et al*., 2009; 2010; De Respinis *et al*., 2010). PCR-RFLPs has been used to distinguish between several *Fusarium* species (Edel *et al*., 1997; Llorens *et al*., 2006; Suga *et al*., 2008)

The association of FGSC species with maize, wheat and barley in South Africa has been demonstrated by Boutigny *et al*. (2011). Crop rotation with wheat and maize under irrigation in South Africa, however, raises concerns about potential increased disease incidence and mycotoxin contamination of these crops due to inoculum build-up. Therefore, information regarding the prevalence of FGSC species on wheat and maize, specifically produced in rotational systems within South Africa, is required. In **Chapter 3**, the occurrence of FGSC species in South African fields where wheat and maize are planted in rotation was investigated. Wheat heads with typical FHB symptoms and maize ears showing symptoms of GER were collected, and the FGSC species associated with these diseases were identified. Their TCT chemotypes were determined using *Tri* gene markers, and a subset of isolates were selected for the evaluation of toxin production *in vitro*.

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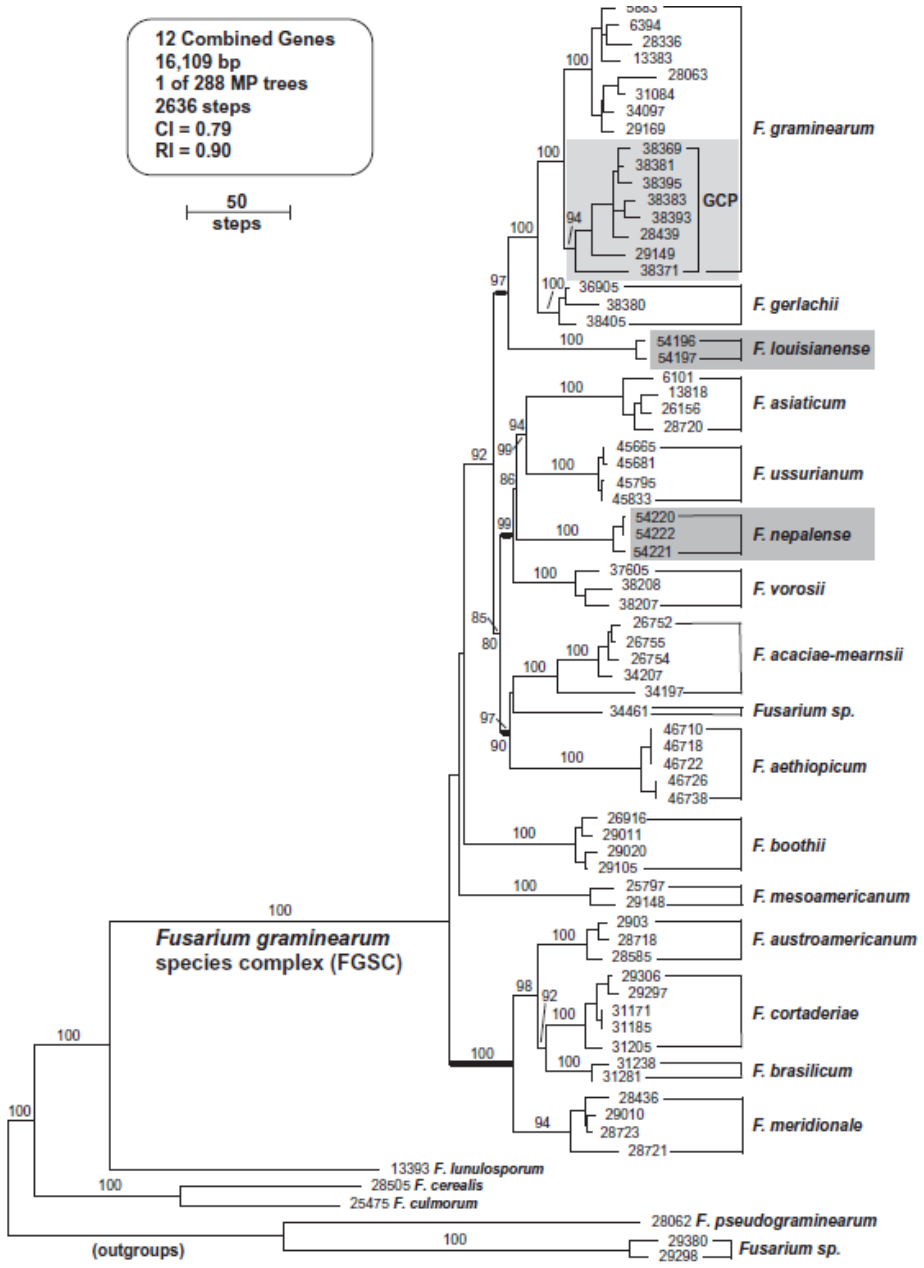

Figure 1. A phylogenetic tree displaying the 16 distinct species within the *Fusarium graminearum* species complex (FGSC) (Sarver *et al*., 2011)

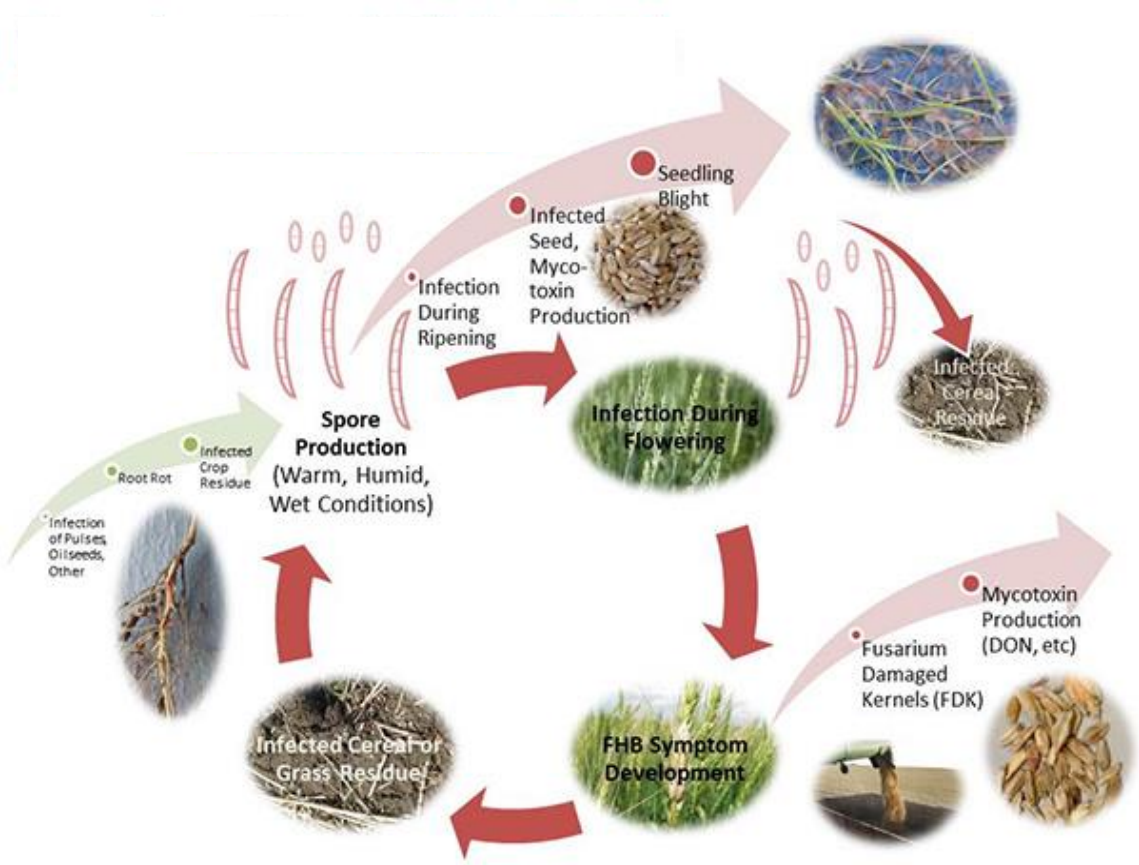

Figure 2. The Fusarium head blight (FHB) disease cycle depicting infection of wheat by FGSC species (http://www.agriculture.gov.sk.ca/fusarium-head-blight).

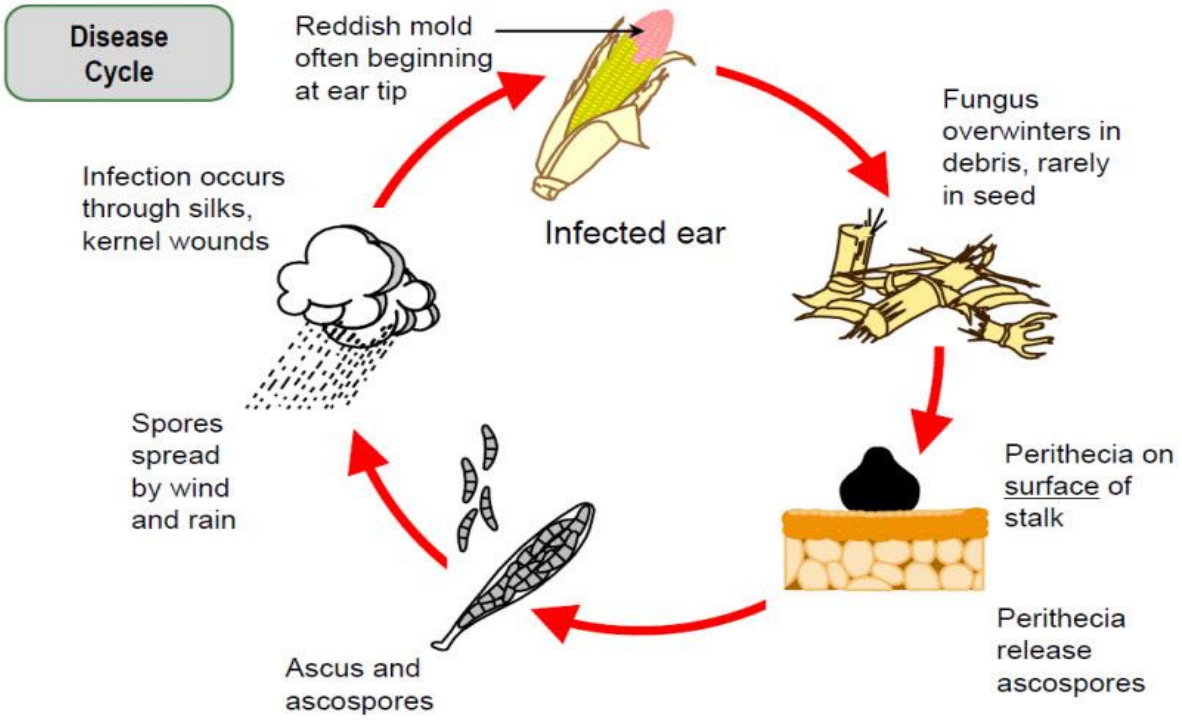

Figure 3. The disease cycle of FGSC species causing Gibberella ear rot on maize (www.pioneer.com/home/site/ca/agronomy/crop-management/corn-insect-disease/gibberella-ear-rot/).

Figure 4. Fusarium head blight of wheat characterised by the loss of chlorophyll and bleaching of the spikelets (photo provided by Ilze Beukes).

Figure 5. Maize ear exhibiting Gibberella ear rot which is characterised by the pink discoloration of the kernels as well as the presence of white and pink mycelia growing from the maize silks towards the base of the maize ear (http://www.agweb.com/article/managing_rotten_corn_an_overview_of_ear_rots/).

CHAPTER 2

The development and evaluation of molecular techniques to identify phylogenetic species within the *Fusarium graminearum* species complex

ABSTRACT

The *Fusarium graminearum* species complex (FGSC) consists of 16 phylogenetic species that cannot be distinguished from each other based on morphological characteristics. The FGSC primarily affects cereal crops and cause diseases such as Fusarium head blight (FHB) of wheat and Gibberella ear rot (GER) of maize. Both diseases result in economic losses due to the reduction in crop yield and grain quality, which includes the contamination of grain with toxic mycotoxins. Six FGSC species; namely *F. graminearum* s.s., *F. cortaderiae*, *F. acaciae-mearnsii*, *F. meridionale*, *F. boothii* and *F. brasilicum* have been documented in South Africa. In this study, matrix assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS),polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) and *F. graminearum* species-specific assay were evaluated as potential techniques to identify FGSC species. Reference isolates of FGSC species occurring in South Africa were used to produce protein mass spectra and restriction digest profiles that distinguish them from one another. Different protein mass spectra were obtained for each of the FGSC species after culturing conditions for MALDI-TOF MS were optimised. Six of the 10 restriction enzymes used to digest the *translation elongation factor α-1* (*EF*-1α) gene region produced restriction profiles that distinguished *F. graminearum* s.s., *F. cortaderiae* and *F. acaciae-mearnsii* from one another. The double restriction digest with *Bfa*I and *Bsa*HI distinguished *F. graminearum* s.s., *F. cortaderiae* and *F. acaciae-mearnsii* from each other and from the remaining two FGSC species evaluated in this study. The restriction profile produced by *Ear*I, when the *histone* (*H*3) gene region was digested, distinguished only *F. boothii* from the FGSC species evaluated. The species-specific PCR could differentiate between *Fusarium graminearum* s.s. and *F. acacia-mearnsii*. Furthermore, *F. cortaderiae* and *F. meridionale* could also be differentiated from the other FGSC species but not from one another while *F. boothii* did not produce a consistent amplification product. These results indicate that MALDI-TOF MS, PCR-RFLPs and the *F. graminearum* species-specific assay can differentiate among FGSC species in South Africa.

INTRODUCTION

Wheat and maize are two of the most important cereal crops grown around the world (FAOSTAT, 2012). Both crops form part of the staple diet of Africans, but maize is more widely planted on the continent (Shephard *et al*., 2007; Shephard, 2008). Wheat and maize production, however, is often constrained by fungal pathogens such as the *Fusarium graminearum* species complex (FGSC), which cause Fusarium head blight (FHB) and Gibberella ear rot (GER), respectively (Windels, 2000). FHB and GER have been associated with reduced grain yields and poor grain quality (McMullen *et al*., 1997; Windels, 2000; Suga *et al*., 2008). The FGSC also contaminates wheat and maize grain with mycotoxins which are associated with health concerns in both humans and animals (Windels, 2000; Bennett and Klich, 2003; Suga *et al*., 2008). The rapid and accurate identification of FGSC is thus important for the management of FHB, GER and their associated mycotoxins.

Sixteen phylogenetic species have been identified within the FGSC which are morphologically difficult to distinguish (O'Donnell *et al*., 2000). These species, therefore, have to be identified using methods based on molecular and cellular differences. PCR- and DNA sequence-based techniques are often used to identify fungi (Hibbett *et al*., 2007). Species within the FGSC, for instance, have been identified by using genealogical concordance phylogenetic species recognition (GCPSR) and multilocus genotyping assay (MLGT) (Ward *et al*., 2008). GCPSR is based on phylogenetic analysis of several gene regions (O'Donnell *et al*., 2000), while MLGT assay is based on the use of probes that target single nucleotide polymorphisms within these genes (Ward *et al*., 2008). Although MLGT is highly accurate in distinguishing species of the FGSC, it is expensive to establish and thus not always feasible. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), a technique based on the presence of natural variation (polymorphisms) within the DNA sequence (Wang *et al*., 2011), is a more feasable and affordable method to study diversity in morphologically indistinguishable fungi. The technique has often been used to investigate the diversity within and among *Fusarium* species (Suga *et al*., 2008; Kemptner *et al*., 2009).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was introduced in the late 1980's for the identification of large biomolecules, such as proteins, in clinical laboratories (Tanaka *et al*., 1988). The MALDI-TOF MS method analyses the protein mass spectra from intact cells, or the extract of cells, which are then compared to known reference mass spectra (Alanio *et al*., 2011). The spectra may also be compared to one another to determine differences between or within species. MALDI-TOF MS has often been widely used for the identification of bacteria amd clinical important filamentous fungi (Chen and Chen, 2005; Dong *et al*., 2009; Sitterle *et al*., 2014; Patel,

2015). This is because the cell structures of fungi are more complex than that of bacteria, and consist of polysaccharides (including chitin) and molecules such as proteins, lipids, peptides and inorganic salts. Several factors involved in the pre-treatment of samples may also affect the quality of the protein mass spectra. These include the growth media, washing procedure, matrix and sample preparation applied (Kemptner *et al*., 2009).

Species specific PCR assays have been used for the identification of *Fusarium* species including *F. acuminatum Ellis & Everh*, *F. avenaceum (Fr.) Sacc., F. culmorum* (W.G. Smith)*, F. poae* (Peck) Wollenweber and *F. graminearum* Schwabe (Parry and Nicholson, 1996; Schilling *et al*., 1996; Doohan *et al*., 1998; Nicholson *et al*., 1998; Williams *et al*., 2002; Waalwijk *et al*., 2003; 2004). Species-specific primers have also been developed for the rapid identification of *Fusarium* species and their mycotoxins (González Jaèn *et al*., 2004; Mulé *et al*., 2005). As the *translation elongation factor 1-α* (*EF*-1α) gene region contains a particularly high number of polymorphisms; this region has often been explored to develop species-specific primers for *Fusarium* species (Rahjoo *et al*., 2008). Nicholson *et al*. (1998) developed the primers for the differentiation of *F. graminearum* from *F. culmorum*. Using these primers, Waalwijk *et al*. (2003) were able to separate four FGSC species; *F. graminearum*, *F. meridionale* Aoki, Kistler, Geiser *et* O'Donnell, *F. asiaticum* O'Donnell, Aoki, Kistler *et* Geiser and *F. austro-americanum* Aoki, Kistler, Geiser *et* O'Donnell; based on their PCR product sizes.

The five FGSC species identified in South Africa produce different mycotoxins in wheat and maize grain. It is, therefore, important to be able to accurately distinguish these from one another. For this reason, MALDI-TOF MS and PCR-RFLPs were evaluated for the identification of morphologically related FGSC species in the country. A *F. graminearum* species-specific PCR (Nicholson *et al*., 1998) was further used to determine whether it produced different band sizes for the FGSC species found in South Africa.

MATERIALS AND METHODS

MALDI-TOF MS biotyping

Fungal isolates: Isolates representing five of the six phylogenetic species within the FGSC that were previously reported in South Africa by the Boutigny *et al*. (2011) were used for the optimisation of the MALDI-TOF MS technique. These include *F. graminearum* s.s., *F. cortaderiae* O'Donnell, T. Aoki, Kistler *et* Geiser, *F. acaciae-mearnsii* O'Donnell, Aoki, Kistler *et* Geiser, *F. meridionale* and *F. boothii* O'Donnell, Aoki, Kistler *et* Geiser(Table 1). *Fusarium brasilicum* Aoki, Kistler, Geiser *et,* O'Donnell was not included, as only one isolate of this FGSC species was collected in South Africa. *Fusarium* species commonly associated with ear rot diseases of maize; such as *Fusarium verticillioides* (Sacc.) Nirenberg, *F. proliferatum*

(Matsushima) Nirenberg and *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas; as well as an isolate of *F. lunulosporum* collected from wheat in South Africa, were also included in the study. *Ilyonectria liriodendri* was included as non-*Fusarium* fungal species (Table 1). The isolates are all maintained at the culture collection of the Department of Plant Pathology at Stellenbosch University in South Africa.

Culturing conditions: Isolates of *F. graminearum* s.s., *F. meridionale, F. boothii*, *I. liriodendri, F. verticillioides* and *F. proliferatum* were first used to optimise the MALDI-TOF MS technique. They were grown on two culture mediums: malt extract agar (MEA) and potato dextrose agar (PDA). The PDA further either contained streptomycin sulphate (40 mg L^{-1}), or was prepared without the antibiotic. The cultures were then incubated for 1 to 10 days, either under 24 hr light or under 24 hr dark conditions, at 25˚C. Samples for MALDI-TOF MS analysis were collected daily. Samples subjected to the different variables tested were evaluated with MALDI-TOF MS analysis, for the determination of the optimal culturing conditions required fpr the production of protein mass spectra. After the optimization of the culturing conditions, all the FGSC isolates were cultured on MEA for 6 days under 24 hr light at 25˚C, while *F. verticillioides, F. proliferatum, F. lunulosporum* and *F. subglutinans* were plated on MEA and PDA for 6 and 10 days under 24 hr light at 25˚C.

Sample preparation: Samples for MALDI-TOF MS analysis were subjected to the Bruker Daltonik MALDI Biotyper cultivation and sample preparation protocols for filamentous fungi according to the manufacturer's recommendations. Fungal mycelia without spores were harvested from the outer margin of the cultures with a sterile toothpick and placed in a sterile microcentrifuge tube. Three samples were collected for each of the treatments. The harvested mycelia were then suspended in 1 mL deionized water, vortexed for 1 min and centrifuged for 2 min at 13 000 revolutions per minute (rpm). The washing water was discarded, and the washing step repeated with a 5-min centrifuge step at 600 rpm. After the washing water was discarded, the washed mycelia were suspended in 300 µL deionized water, followed by the addition of 900 µL absolute ethanol. The suspension was then thoroughly vortexed, centrifuged for 2 min at 13 000 rpm, and the supernatant discarded. The resultant pellet was dried and placed in a SpeedVac at 37˚C for 10 min. The dried pellet was again suspended in 60 µL of 70% formic acid and 60 µL of acetonitrile, and mixed by pipetting. The sample was then vortexed and centrifuged for 2 min at 13 000 rpm.

For MALDI-TOF MS analysis, samples (1 µL droplets) were spotted directly onto the 384 MTP MALDI polished steel target plate (Bruker-Daltonik GmbH, Bremen, Germany), in triplicate, and air-dried. The spotted samples were then coated with 2 µL of alpha-cyano-4-hydroxy-cinnamic acid matrix (α-CHCA) (10 mg mL^{-1}) (Bruker-Daltonik GmbH). A bacterial

test standard (BTS- *Escherichia coli*) (1 µL droplet) was also spotted onto the calibration position of the MALDI target plate for quality control and quality assurance. The samples were analysed on the UltrafleXtreme MALDI Tof/Tof (Bruker Daltonics) mass spectrometer at the facilities of the Department of Biotechnology, University of the Western Cape, Cape Town.

MALDI-TOF MS analysis: Protein mass spectra were automatically collected in the linear ion, positive mode within a protein mass range from 3 000 to 15 000 Dalton (Da). The initial laser energy was set to 50%, with a maximum laser power of 80% to obtain peaks. Spectrum signal acquisition was completed with the FlexControl (version 3.4; Bruker Daltonics, Bremen, Germany) in auto-execute mode by pulsating the target spot. Each spectrum was collected 10 times with a laser frequency of 1 000 Hertz (Hz). Peptide spectra of accumulated 3 000 shots were processed with the software package MALDI Biotyper Real Time Classification (Bruker Daltonics). The software performs steps of smoothing and baseline correction during peak evaluation, and the peak resolution was set to be larger than 400 for the identification of the most significant peaks.

PCR – restriction fragment length polymorphism

Fungal isolates: At least two isolates representing each of the five FGSC species previously reported in South Africa; *F. graminearum* s.s., *F. cortaderiae, F. acaciae-mearnsii, F. meridionale* and *F. boothii*; were selected for PCR-RFLP analysis. Additionally, two isolates each representing three other *Fusarium* species; namely *F. verticillioides, F. proliferatum and F. subglutinans*; and one isolate representing *F. lunulosporum*, was included as negative controls (Table 1).

Culture conditions and DNA extraction: All isolates were grown on PDA amended with streptomycin sulphate (40 mg L^{-1}) under 24 hr fluorescent light at 25˚C. Fungal mycelia were harvested after 6 days and transferred into a 2-mL microcentrifuge tube containing 400 µL lysis buffer and glass beads, for the extraction of genomic DNA with the Wizard ® SV Genomic DNA Purification System (Promega, Wisconsin, U.S.A). The samples were shaken for 10 min at a frequency of 30 Hz in a mixermill MM301 (Retsch GmbH, Mettmann, Germany), and thereafter incubated in a water bath for 30 min at 65˚C. The samples were then centrifuged for 8 min at 14 000 rpm, and the lysate was transferred to a mini-column and collection tube assembly. Samples were centrifuged for 3 min at 12 000 rpm and the liquid in the collection tubes were discarded. The mini-column was washed with 650 µL washing solution, and centrifuged again for 1 min at 12 000 rpm. This step was repeated three more times. The final washing step of the mini-column was followed by centrifugation

for 2 min at 12 000 rpm. The mini-column was then transferred to a 1.5-mL micro-centrifuge tube, and 75 µL nuclease-free water was added before incubation for 2 min. This step was repeated twice. The mini-column micro-centrifuge assembly was then centrifuged for 1 min at 12 000 rpm. The mini-column was discarded and 1.2 µL RNase was added to each sample. The samples were then vortexed and incubated for 10 min at room temperature. DNA concentration and quality was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific, South Africa), and the DNA stored at 4°C.

Amplification of the translation elongation factor *1-α and* histone *3 gene regions*: The *EF*-1α gene region was amplified with the EF1 and EF2 primers (O' Donnell *et al*. 1998; Geiser *et al*. 2004), and the *H*3 gene region with the H3dStyI (Suga *et al*., 2008) and H3R1 primers (O'Donnell *et al*., 2004) (Table 2). The *EF*-1α and *H*3 amplification was performed using 20 ng of fungal DNA in a total reaction mixture of 25 µL and 20 µL, respectively. The *EF*-1α PCR reaction consisted of 0.2 µM of each primer, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.24 mM, dNTP, 1.25 µL of 20 mg mL^{-1} bovine serum albumin (BSA), and 0.3 U *Taq* DNA polymerase. The *H*3 reaction mixture consisted of 0.4 µM of each primer as well as 2x KAPA Ready mix with Mg^{2+} (Kapa Biosystems, Cape Town, South Africa). The samples were subjected to thermal cycling with a GeneAmp® PCR System 9700 (Applied biosystems, California, United States). Thermal settings for the amplification of the *EF*-1α gene region consisted of 5 min at 94°C; followed by 35 cycles for 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C; before the final step for 7 min at 72°C. The thermal cycle conditions for *H*3 consisted of an initial step for 2 min at 95°C; followed by 35 cycles for 30 sec at 95°C, 45 sec at 56°C and 45 sec at 72°C; with a final step for 2 min at 72°C. The PCR products were separated by gel electrophoresis on a 1% agarose gel, stained with GrGreen (Inqaba Biotec), and visualised under a UV transilluminator.

Sequencing and bioinformatics: The PCR products of the FGSC isolates, for both the *EF*-1α and *H*3 gene regions, were purified with the MSB® Spin PCRapace Kit (Invitek, Rhineland-Palatinate, Germany) according to the manufacturer's recommendations. The purified PCR products were submitted to the Central Analytical Facility (CAF) of the Stellenbosch University, South Africa for sequencing with both the forward and reverse primers. DNA sequences were edited, aligned, and restriction enzymes unique to FGSC species identified using Geneious Pro 5.3.3 (Biomatters Ltd., Auckland, New Zealand) which predicted the restriction enzymes associated with the restriction site.

Restriction digests: For PCR-RFLP analysis of the *EF*-1α gene region, 10 µL of the PCR products were digested with 10 restriction enzymes in a total reaction volume of 20 µL

(Table 3) according to the manufacturer's' recommendations. For digestion of the *H*3 gene region, 15 µL of the PCR product was mixed with the restriction enzymes *Eco*RV, *Ear*I and *Tru*1I in total reaction volumes of 30, 50 and 30 µL, respectively, according to the manufacturer's recommendations. The restriction fragments obtained were visualised by electrophoresis on a 2% agarose gel stained with GrGreen (Inqaba Biotec) for 2 hr at 75 V. The 100-bp GeneRuler DNA ladder (Thermo Scientific) was included as a size standard to determine fragment size. At least two independent experiments were performed per enzyme.

Double restriction digests: Double restriction digests were carried out with restriction enzymes *Bfa*I and *BsaH*I. The digest reaction consisted of 15 µL of the *EF*-1α PCR product, 10 U of each enzyme and 1x Cutsmart buffer in a 50 µL reaction volume. The reaction mixture containing the restriction enzymes *Bfa*I and *BsaH*I were incubated for 1 hr at 37°C, followed by an inactivation step for 20 min at 80°C. The PCR products were separated for 2 hr at 75 V on a 2% agarose gel that contained GrGreen stain (Inqaba Biotec). The molecular size of each fragment was determined by using the 100-bp GeneRuler DNA ladder (Thermo Scientific).

Fusarium graminearum species-specific PCR

The *F. graminearum* s.l.-specific PCR of Nicholson *et al*. (1998) was evaluated on five FGSC and four *Fusarium* species collected from wheat and maize in South Africa (Table 1). Amplification of the hypothetical gene was conducted by using 20 ng of DNA in a total reaction volume of 25 µL, consisting of 1x buffer, 3 mM $MgCl_2$, 0.4 nM dNTPs, 1.25 µL of 20 mg mL^{-1} BSA, 0.2 U taq DNA polymerase and 5 µM of each primer (Table 2). Amplification conditions were as follows: denaturation at 2 min at 94°C; followed by 35 cycles of 45 sec at 94°C, 30 sec at 58°C and 45 sec at 72°C; with a final extension step of 5 min at 72°C (Nicholson *et al*., 1998). The PCR products were separated on a 1% agarose gel stained with GrGreen (Inqaba Biotec) for 90 min at 75 V. The molecular size of each fragment was determined by using the 100-bp GeneRuler DNA ladder (Thermo Scientific).

RESULTS

MALDI-TOF MS biotyping

The FGSC species *F. graminearum* s.s., *F. meridionale* and *F. boothii* produced protein spectra that allowed for the differentiation between FGSC species. The culturing of FGSC species on MEA produced protein mass spectra with more distinct peaks of greater intensities (peak intensities >1.0 x 10^4 *Intens*. [*au*.]) than when the species were cultured on PDA (Fig. 1). Protein spectra with low peak intensities (peak intensities <0.4 x 10^4 *Intens*.

[*au*.]) and few distinct peaks were produced when FGSC species were grown on PDA amended with streptomycin (Fig. 2). FGSC species cultured on PDA media for 1-5 and 7-10 days, respectively, produced protein spectra with few peaks and low intensities compared to species cultured on PDA media for 6 days (Fig. 3 and 4). When *F. verticillioides* and *F. proliferatum* were cultured on MEA and PDA, however, they produced more peaks with higher intensities after 6 and 10 days, respectively, than compared to the other days evaluated (Fig. 5). The number of peaks and their intensities were not influenced by light (data not shown). The optimal culture conditions for FGSC species were on MEA at 25°C under constant light for 6 days, while the optimal conditions for *F. verticillioides* and *F. proliferatum* were on MEA and PDA under constant light at 25°C for 10 days.

The extraction protocol resulted in the generation of adequate protein spectra to distinguish among FGSC species. The spectra exhibited peaks ranging between 2 000 and 13 000 molecular mass to charge (m/z) ratio. Protein mass spectra generated for non-FGSC species (*F. verticillioides*, *F. proliferatum, F. subglutinans* and *F. lunulosporum*) were different when compared to the profiles generated for all five FGSC species (produced different peaks at different intensities). The five FGSC species generated protein mass spectra which enabled differentiation among FGSC and with other *Fusarium* species (Fig. 6). The protein mass spectra of *F. boothii* showed three distinguishing protein peaks (4888.678, 9122.717 and 11648.857 m/z). The protein mass spectra of *F. boothii* and *F. meridionale* showed eleven peaks with a single peak (11018.815 m/z) uniquely present in *F. meridionale*. Nine protein peaks were produced by *F. acacia-mearnsii* with a single protein peak (4148.413 m/z) exclusively associated with this FGSC species. The protein mass spectra produced for *F. cortaderiae* had 7 protein peaks with protein peak 9206.683 m/z uniquely associated with *F. cortaderiae*. Twelve protein peaks were produced by *F. graminearum* s.s. with protein peaks 4702.785, 8405.514, 9404.096, 10700.443, 11588.840 and 13785.467 m/z exclusively identified for *F. graminearum* s.s. (Fig. 6). The MALDI-TOF protein spectra produced for the same species, similarly cultivated, were reproducible following two independent experiments.

PCR – restriction fragment length polymorphism

The amplification of the *EF*-1α gene of FGSC reference isolates; as well as *F. verticillioides*, *F. proliferatum, F. subglutinans and F. lunulosporum*; resulted in an 800-bp amplification fragment. The amplification of the *H*3 gene region of the FGSC reference isolates and *F. lunulosporum* produced a 200-bp fragment. No amplification fragments for the *H*3 gene region were obtained for *F. verticillioides*, *F. proliferatum* and *F. subglutinans*.

Ten restriction recognition sites were identified within the *EF*-1α gene region for FGSC species. The restriction enzymes included *BsaH*I, *Tru*1I, *Dra*I, B*seG*I, *Bfa*I, *Spe*I,

*Sma*I, *Rsa*I, *Hpy*188III and *TspR*I (Table 3). Six of the 10 restriction enzymes, *Bsa*HI, *Tru*1I, *Dra*I, B*seG*I, *Bfa*I and *Spe*I, produced restriction fragments. Digestion of *EF*-1α with *Bsa*HI produced unique restriction fragment profiles for two FGSC species, namely *F. graminearum* s.s. and *F. cortaderiae* (Fig. 7). The partial restriction digest produced three fragments with sizes of 800 (undigested), 200 and 300 bp for *F. cortaderiae*. The restriction fragment profile representing *F. graminearum* s.s. consisted of two fragments >300 bp and 400 bp in size. The restriction enzymes *Tru*1I, *Dra*I and *Bse*GI distinguished *F. cortaderiae* from the other four FGSC species (Fig. 8-10). Enzymes *Tru*1I and *Dra*I generated fragment sizes of approximately ~ 700 bp, respectively, whereas digestion with *Bse*GI produced a banding pattern consisting of two fragments of 350 bp and 700 bp in size. Unique fragment profiles were produced for *F. acacia-mearnsii* when the *EF*-1α gene region was digested with enzymes *Bfa*I and *Spe*I (Fig. 11 and 12). Both restriction enzymes generated fragment patterns characterised by two fragments of <300 and 500 bp. Four restriction enzymes (*Bsa*HI, *Tru*1I, *Dra*I and *Spe*I) did not digest the *EF*-1α gene region amplified from *F. verticillioides, F. proliferatum, F. subglutinans* and *F. lunulosporum.* Digestion with *Bse*GI produced two fragments (~200 and 400 bp) for *F. verticillioides* only, while the digestion with enzyme *Bfa*I produced two fragments (200 and 800 bp) for *F. lunulosporum*.

Double restriction digests of the amplified *EF*-1α gene region with the restriction enzymes *Bfa*I and *BsaH*I (Fig. 13) resulted in the differentiation of three of the five FGSC species evaluated, namely *F. graminearum* s.s., *F. cortaderiae* and *F. acacia-mearnsii. Fusarium cortaderiae* was represented by three fragment patterns (200, 300 and 800 bp), *F. graminearum* s.s. by two fragments approximately >300 and 400 bp and *F. acacia-mearnsii* produced a fragment pattern consisting of two fragments (<300 and 800 bp). The double restriction digest did not result in the digestion of the *EF*-1α amplification product of *F. lunulosporum* or any of the other non-FGSC species tested.

The restriction enzymes identified with restriction recognition sequences in the *H3* gene region were *EarI* and *Tru*1I. Digestion of the *H*3 amplification product with *Ear*I (Fig. 14) resulted in the identification of *F. boothii,* which remained undigested and represented by a 200-bp fragment. Digestion with *Tru*1I generated a fragment of <200 bp to identify *F. acacia-mearnsii* (Fig. 15), and *Eco*RV produced a fragment of <200 bp to identify *F. graminearum* s.s. (Fig. 16). The *H*3 gene region primers amplified *F. lunulosporum,* but *Tru*1I and *Eco*RV did not digest the amplified PCR product. The *H3* gene region of *F. lunulosporum* was digested by *EarI* into a fragment <200 bp.

Fusarium graminearum species-specific PCR

Amplification products of the hypothetical gene confirmed the identity of the FGSC reference isolates as phylogenetic species. *Fusarium graminearum* s.s. isolates produced a single

400-bp product, as was previously reported by Nicholson *et al*. (1998). Species previously identified as *F. acacia-mearnsii*, however, produced a 300-bp PCR product, whereas *F. cortaderiae* and *F. meridionale* both generated a 500-bp PCR product (Fig. 17). The primers did not consistently amplified *F. boothii*. Amplification of *F. lunulosporum* generated a banding pattern with two fragments of approximately 400 and 150 bp. The isolates representing *F. verticillioides*, *F. proliferatum* and *F. subglutinans* did not amplify with the *F. graminearum* species-specific primer set.

DISCUSSION

Species of the FGSC on wheat and maize have a global distribution (O' Donnell *et al*., 2000, 2004; Láday *et al*., 2004; Ramirez *et al*., 2007; Lee *et al*., 2009; Desjardins *et al*., 2011). Species such as *F. asiaticum* dominates the Chinese population of FGSC because of their reported greater fitness in the region (Qu *et al*., 2008), whereas *F. graminearum* s.s. dominates in Europe (Láday *et al*., 2004). In South Africa, *F. boothii* has primarily been associated with maize ears, because of their expected preference for the crop (Boutigny *et al*., 2011). Some FGSC species, such as *F. meridionale*, produces nivalenol, whereas others such as *F. graminearum* s.s. and *F. boothii* primarily produces deoxynivalenol (Starkey *et al*., 2007). It is, thus, important to be able to distinguish these species from each other. To date, only MLGT assay has been used to accurately identify FGSC species (O'Donnell *et al*., 2000; 2004; 2008; Starkey *et al*., 2007; Ward *et al*., 2008). In this study, three additional techniques were identified that could be used to distinguish among the morphologically-related FGSC species present in South Africa.

MALDI-TOF MS was successfully used to separate the five FGSC species evaluated from each other in this study. MALDI-TOF MS has previously been used to distinguish among *Fusarium* species (Dong *et al*., 2009; Marinach-Patrice *et al*., 2009), but never before to identify *Fusarium* species within species complexes such as FGSC and the *F. equiseti-incarnatum* (syn. *F. equiseti* (Corda) Saccardo) species complex. The protein mass spectra produced by non-FGSC species were distinctly different from that of species within the FGSC and other *Fusarium* species. Using the MALDI-TOF MS fingerprint (absence and or presence of protein peaks in conjunction with the number of peaks) differentiation was possible (Dong *et al*., 2009; Pavlovic *et al.*, 2013). The number of protein peaks produced by *F. graminearum* s.s. when compared to other FGSC species, as well as six distinct protein peaks only produced by *F. graminearum* s.s. enabled its differentiation from the other FGSC species evaluated. The *F. cortaderiae* protein mass spectra had the lowest number of peaks of the five FGSC species. The low peak number as well as the presence of a single distinct protein peak could be used to differentiate *F. cortaderiae* from the remaining FGSC species.

Fusarium boothii and *F. meridionale* had the same number of peaks but differed in distinct protein peaks produced. The value of the technique to differentiate all 16 species within the FGSC needs to be investigated. Additionally, more isolates representing the FGSC species in South Africa could be included in future studies.

In the current study, several factors have influenced the quality of the protein mass spectra produced by FGSC species. These include the growth media used and the age of cultures. It was interesting to find that PDA amended with streptomycin inhibited the production of protein mass spectra, with fewer peaks at lower intensities compared to non-amended PDA. Whether streptomycin inhibited permits the production of metabolites could possibly act as inhibitors need to be further investigated (Santos *et al*., 2010). Previous studies have used PDA and MEA as growth media for the production of mycelia used in the MALDI-TOF evaluation. Potato dextrose agar and MEA allowed for the identification of several clinical important *Fusarium* species (Dong *et al*., 2009). Mycelia harvested from the most actively growing area (outer margin) of 6-day old colonies produced the best material for MALDI-TOF MS. Vegetative growth on agar is characterised by different growth zones, which correspond to different developmental stages of the fungus (Santos *et al*., 2010). It is, thus, associated with characteristics such as pigmentation, which could influence the protein mass spectra. Whereas the protocol evaluated in this study proofed to be efficient for the identification of local FGSC species by means of MALDI-TOF MS, its usefulness in other laboratories and for other FGSC species still needs to be proved.

MALDI-TOF MS requires a spectral library for the identification of unknown microbial samples, for bacteria and medicaly important yeasts such spectral libraries are readily available. Before the technology could be routinely used for filamentous fungal species of economic importance are of concern, many factors should be taken into consideration. Firstly, it is important to determine which components of fungi need to be investigated; mycelia, spores or cellular extracts, this study used mycelia as FGSC species produce spores after several weeks. Secondly, its flexibility needs to be considered, such as its ability to identify closely related and unrelated fungal species, and how much time and preparation is required for samples to be analysed. Finally, costs associated with acquiring and maintaining such equipment, as well as the cost of trained personnel, need to be considered. Future research should include testing the MALDI-TOF MS technique against a panel of unknown isolates.

In contrast to MALDI-TOF MS, PCR-RFLP has been broadly applied for the identification of fungi. In this study, PCR-RFLPs of the *EF*-1α and *H3* gene regions were useful to separate five of the FGSC species present in South Africa. The restriction enzymes able to separate FGSC species were not able to identify all the non-FGSC species. While the technique is an easy and accurate method for the identification of closely related species

(Wang *et al*., 2011), several more gene regions and restriction enzymes will have to be evaluated before it could be used to routinely identify species within the FGSC. The assay is inexpensive and does not require advanced instruments; it is quick to set up and thus can easily be used to identify a large number of samples (González Jaèn *et al*., 2004). PCR-RFLP analysis is, however, relatively time-consuming (González Jaèn *et al*., 2004).

The species-specific primer pair developed for *F. graminearum* s.l. by Nicholson *et al*. (1998) proved to be of good value to differentiate three of the six FGSC species identified in South African grain crops. *Fusarium graminearum* s.s., *F. meridionale*, *F. cortaderiae* and *F. acacia-mearnsii* were amplified with the species specific primers. *Fusarium graminearum* s.s. and *F. acacia-mearnsii* could all be distinguished from one another on the size of the PCR-amplified product, however F*. meridionale* and *F. cortaderiae* produced the same size PCR-amplified product. *Fusarium boothii* was not consistently amplified. Waalwijk *et al*. (2003) also reported the identification of *F. graminearum* s.s. and *F. meridionale* by amplifying their DNA with this primer pair*.* It is not expected that the primer pair will be able to differentiatebetween all the FGSC species, but it could be of considerable value to accurately detect existing FGSC species occurring on South African grain crops.

The accurate and rapid identification of mycotoxigenic fungi contaminating economically important grain crops is vital, and could contribute to the application of appropriate disease management strategies. In this study, three molecular techniques (MALDI-TOF MS, PCR-RFLPs and *F. graminearum* species-specific assay) were shown to be useful for the identification of closely related FGSC species present in South Africa. This is of particular value, as morphological techniques cannot distinguish these FGSC species, while setting up a MLGT assay platform in the country is not economically justifiable. The *F. graminearum* species-specific assay is less labour intensive and relatively cost effective than the other two methods investigated. Therefore the use of the species-specific assay for the routine identification of FGSC species in South Africa would be recommended. MALDI-TOF MS, PCR-RFLPs and *F. graminearum* species-specific assay should, however, be used with care to prevent inaccuracy and false positive identification of closely related FGSC species entering South Africa. Further research should include optimisation and testing of the other FGSC species that have not been reported in South Africa.

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Table 1. *Fusarium graminearum* species complex and other *Fusarium* species used to evaluate Matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) and PCR-restriction fragment length polymorphisms and diagnostic techniques.

Species identity	Isolate number MALDI-TOF MS	Isolate number PCR-based techniques
F. graminearum s.s.[1]	2.547; 2.922; 2.863	2.922; 2.863; 2.894; 2.574
F. cortaderiae[1]	2.571; 2.911	2.551; 2.571; 2.911
F. acaciae-mearnsii[1]	2.899; 2.898	2.899; 2.898; 2.908
F. meridionale[1]	2.905; 2.623	2.895; 2.905; 2.896; 2.853
F. boothii[1]	2.805; 2.968; M0100	2.805; 2.968; 2.629; M0100
F. verticillioides[2]	MRC 826; MRC 8559	MRC 826; MRC 8559
F. proliferatum[2]	MRC 2301; MRC 6903	MRC 2301; MRC 6903
F. lunulosporum[3]	N113	N113
F. subglutinans[2]	MRC 0115; MRC 2293	MRC 0115; MRC 2293
I. liriodendri[4]	C204	C204

[1]Isolates representing species within the *Fusarium graminearum* species complex (FGSC), previously reported in South Africa and identified using MLGT, was used as reference isolates in this study (Boutigny *et al*., 2011)

[2]*Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans* were provided by Dr. H. Vismer (MRC-PROMEC, Tygerberg, South Africa)

[3]*Fusarium lunulosporum* was provided by Gert van Coller (Western Cape Department of Agriculture, South Africa)

[4]*Ilyonectria liriodendri* provided by Shaun Langenhoven (Department of Plant Pathology, Stellenbosch University, South Africa)

Table 2. Gene regions, primer names and sequences used for the molecular identification of *Fusarium graminearum* species complex and other *Fusarium* species.

Target	Primer name	Primer sequence (5'---3')	Reference
EF-1α	EF1	ATGGGTAAGGA(A/G)GACAAGAC	O' Donnell *et al*. (1998); Geiser *et al*. (2004)
	EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	O' Donnell *et al*. (1998); Geiser *et al*. (2004)
*H*3	H3dStyI	AGCATCACCYGAACATCGCATCATCCCATG	Suga *et al*. (2008)
	H3R1	TTGGACTGGATRGTAACACGC	O'Donnell *et al*. (2004)
F. graminearum	Fg16F	CTCCGGATATGTTGCGTCAA	Nicholson *et al*. (1998)
	Fg16R	GGTAGGTATCCGACATGGCAA	Nicholson *et al*. (1998)

Table 3. Restriction enzymes used for the identification of *Fusarium graminearum* species complex and other *Fusarium* species by means of PCR-restriction fragment length polymorphisms.

Target gene region	Enzyme name	Restriction recognition site	Manufacturer or Supplier
EF-1α	*Tru*1I	T^TAA	Thermo Scientific
EF-1α	*Dra*I	TTT^AAA	Thermo Scientific
EF-1α	*Bse*GI	GGATG(2/0)^	Thermo Scientific
EF-1α	*Bfa*I	C^TAG	New England Biolabs
EF-1α	*Spe*I fast digest	A^CTAGT	Thermo Scientific
EF-1α	*Bsa*HI	GR^CGYC	New England Biolabs
EF-1α	*Hpy*188III	TC^NNGA	New England Biolabs
EF-1α	*Tsp*RI fast digest	CASTG(2/-7)	Thermo Scientific
EF-1α	*Sma*I	CCC^GGG	Thermo Scientific
EF-1α	*Rsa*I	GT^AC	Thermo Scientific
*H*3	*Eco*RV	GAT^ATC	New England Biolabs
*H*3	*Ear*I	CTCTTC (1/4)	New England Biolabs
*H*3	*Tru*1I	T^TAA	Thermo Scientific

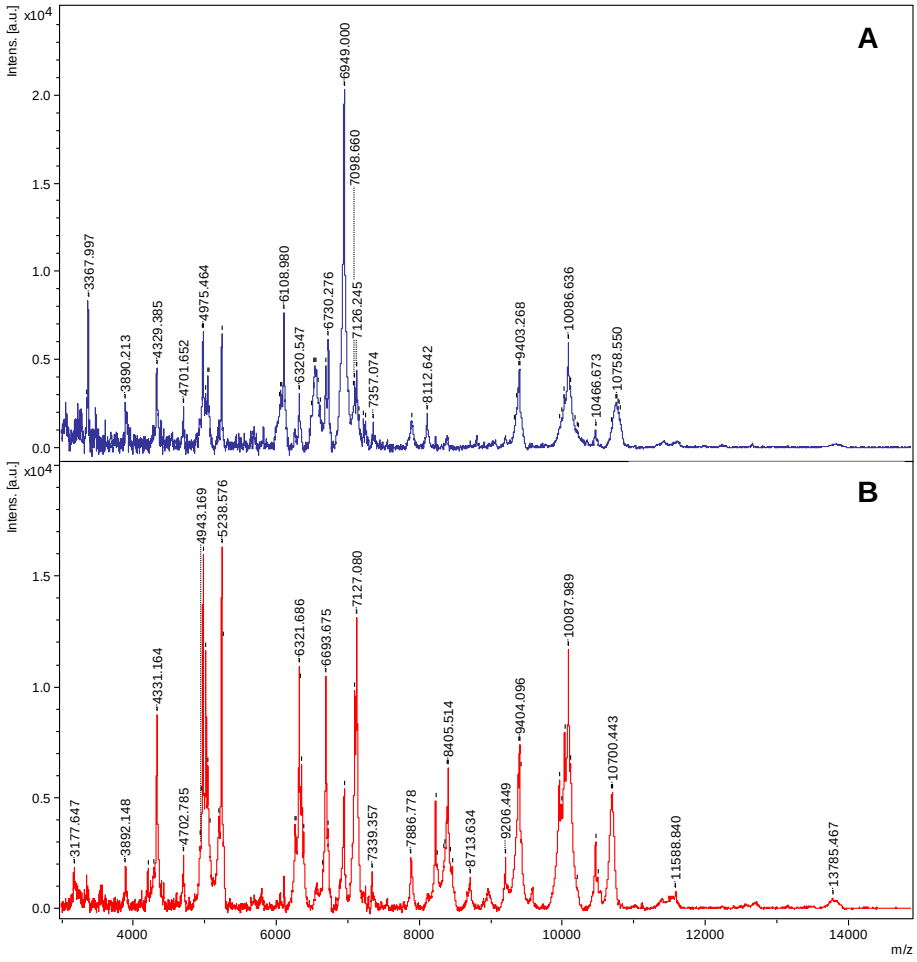

Figure 1. Matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra profiles of *Fusarium graminearum* s.s. grown on (A) potato dextrose agar and (B) malt extract agar.

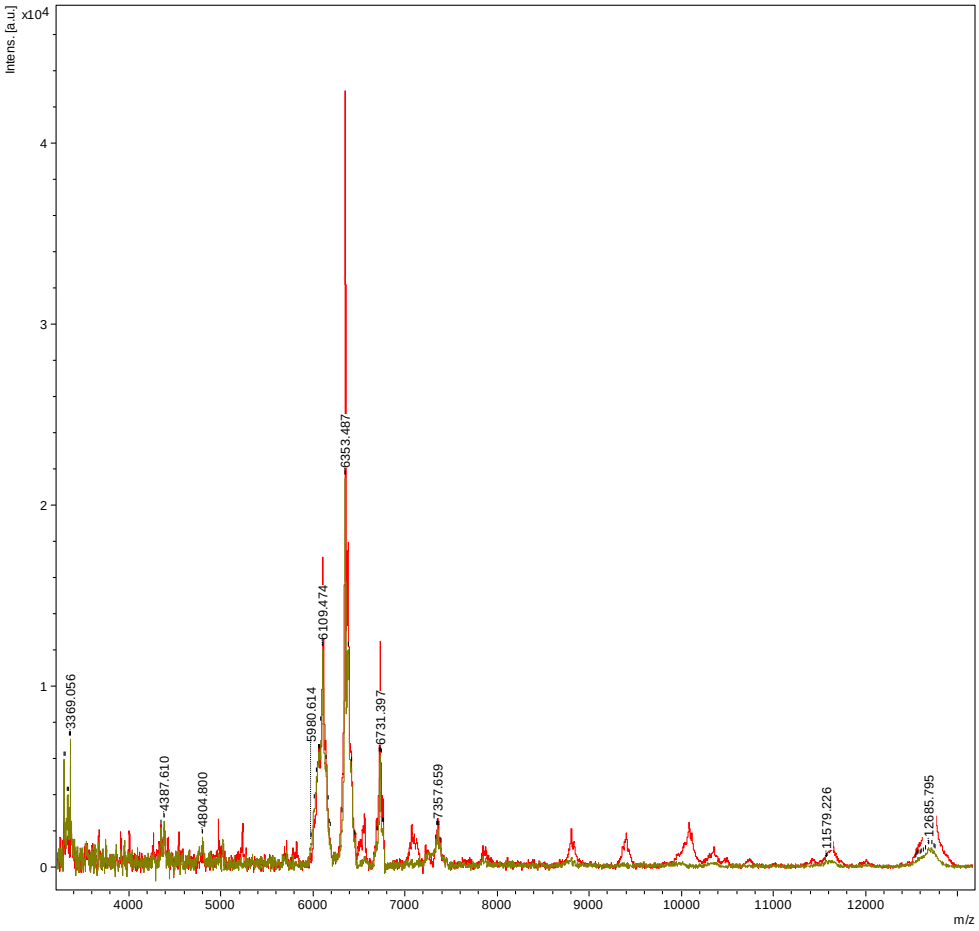

Figure 2. Matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra profiles of *Fusarium graminearum* s.s. grown on potato dextrose agar (PDA) in red and PDA amended with streptomycin in green.

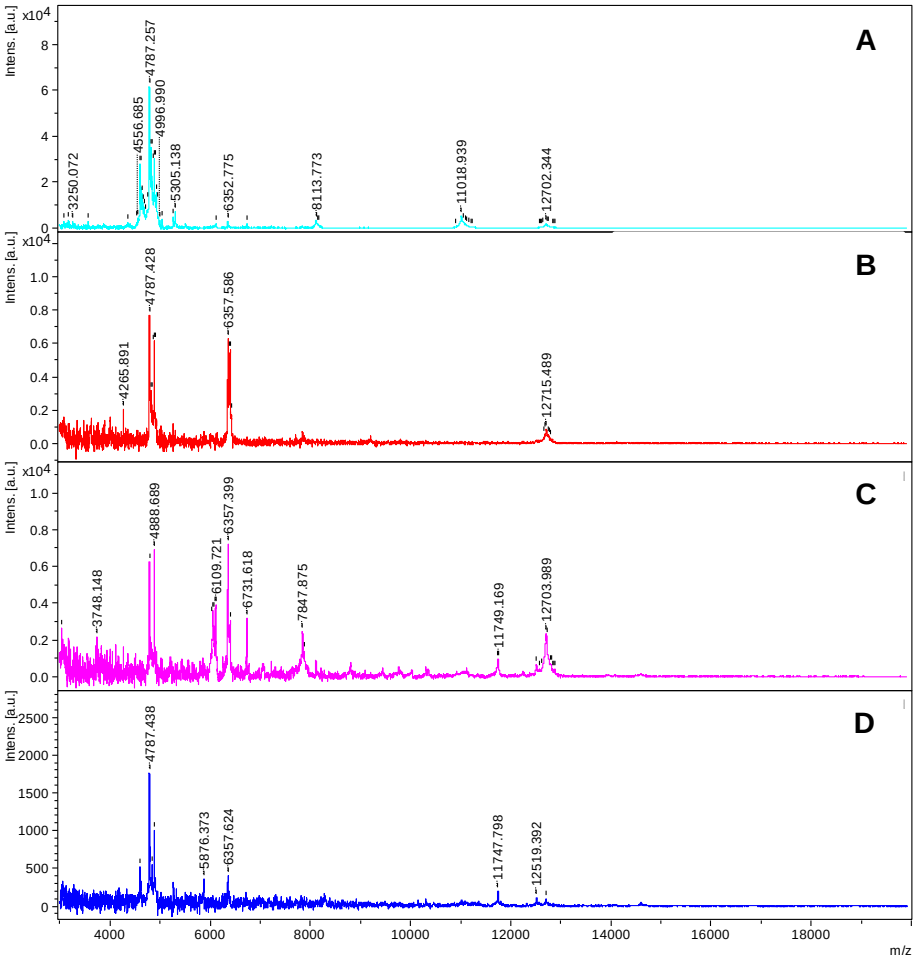

Figure 3. Matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra profiles of *Fusarium meridionale* grown on malt extract agar for (A) 1 (B) 2 (C) 3 and (D) 4 days.

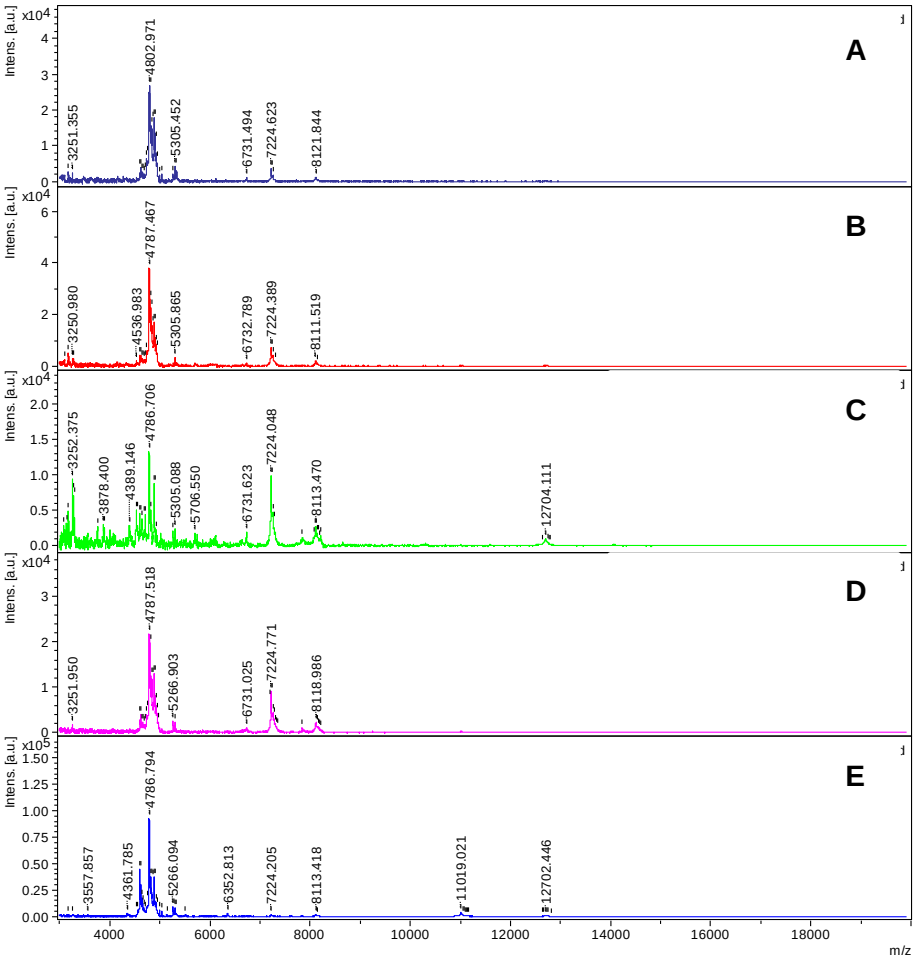

Figure 4. Matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra profiles of *Fusarium meridionale* grown on malt extract agar for (A) 5 (B) 7 (C) 8 (D) 9 and (E) 10 days.

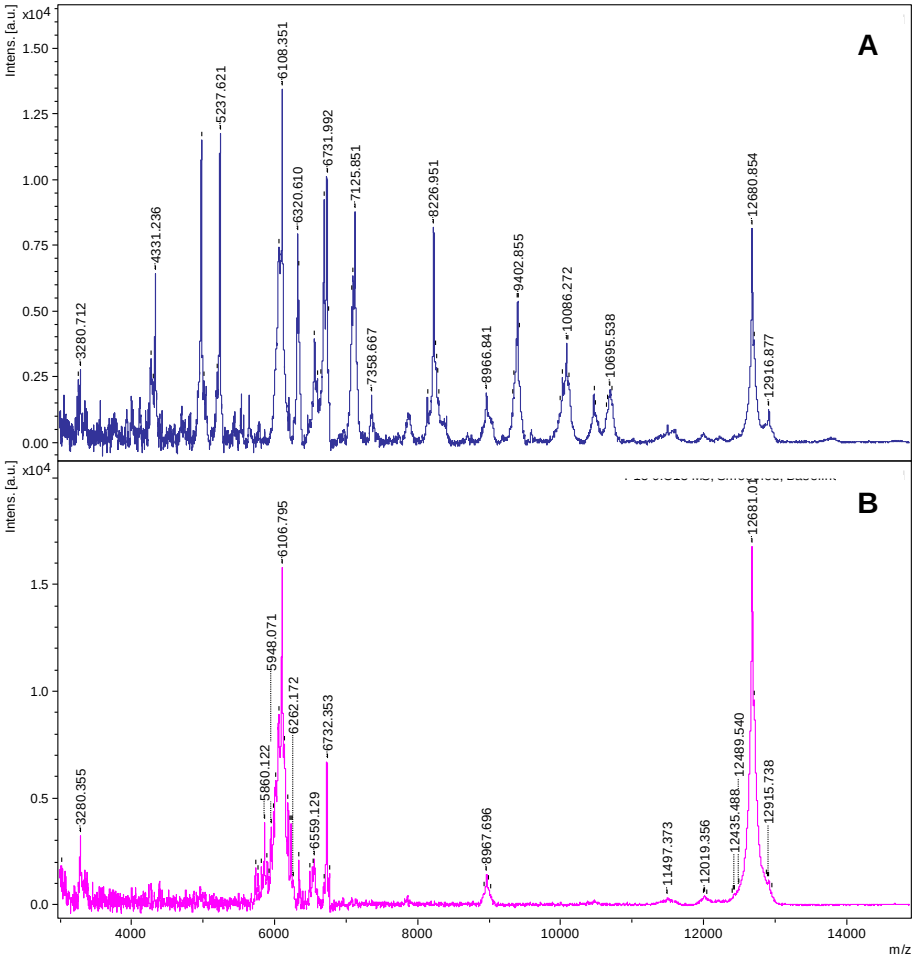

Figure 5. Matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra profiles of *Fusarium proliferatum* following culturing for 10 days on (A) potato dextrose and (B) malt extract agar.

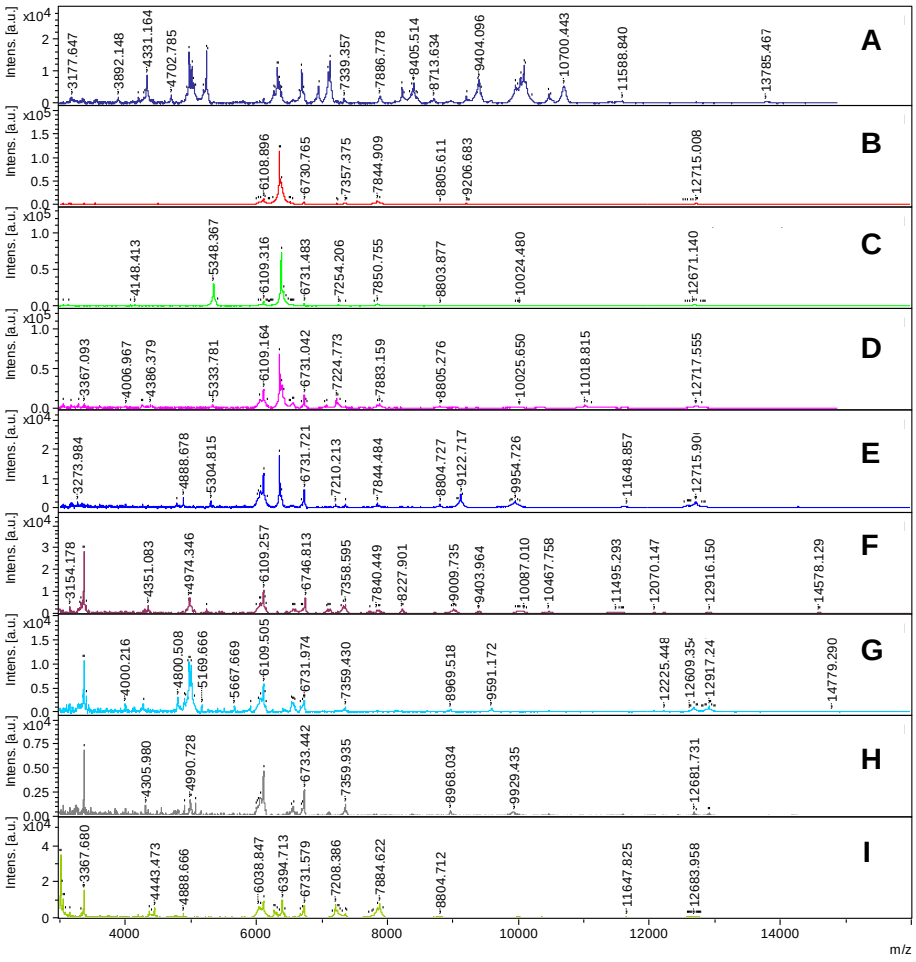

Figure 6. Comparison of matrix assisted laser desorption ionization – time of flight mass spectrometry mass spectra of *Fusarium* species cultivated on malt extract agar for 6 days under 24 hr light at 25˚C and analysed under the same conditions. (A) *F. graminearum* s.s., (B) *F. cortaderiae*, (C) *F. acaciae-mearnsii*, (D) *F. meridionale*, (E) *F. boothii*, (F) F. verticillioides, *(G) F. proliferatum,* (H) *F. subglutinans* and (I) *F. lunulosporum.*

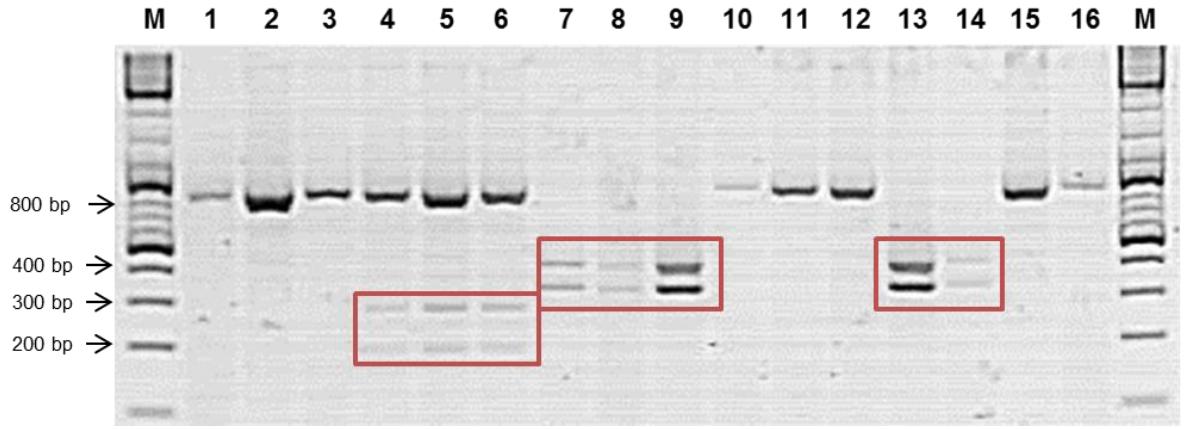

Figure 7. The restriction digest of the translation *elongation factor α-1* (*EF*-1α) region with *Bsa*HI of the *Fusarium* species. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. cortaderiae*, 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. graminearum* s.s., 10. *F. meridionale*, 11. *F. meridionale*, 12. *F. meridionale*, 13 *F. graminearum* s.s., 14. *F. graminearum* s.s., 15. *F. boothii*, 16. *F. boothii*.

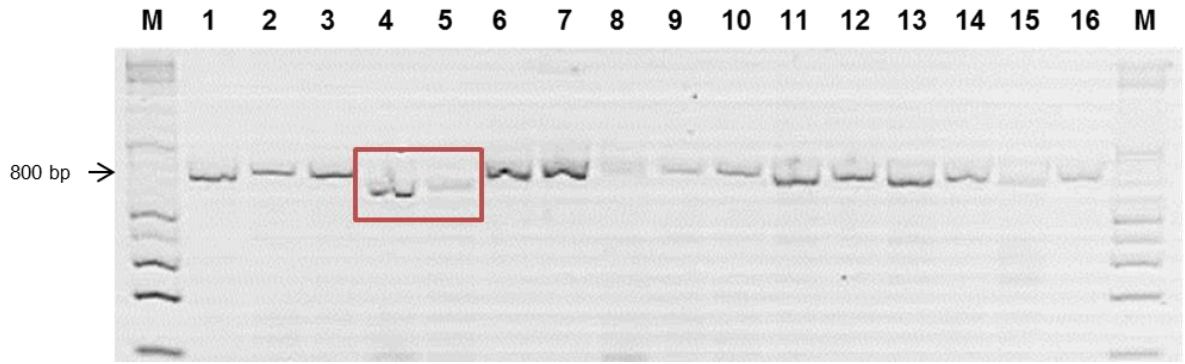

Figure 8. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Tru*1I. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. graminearum* s.s., 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. meridionale*, 10. *F. meridionale*, 11. *F. meridionale*, 12 *F. boothii*, 13. *F. boothii*, 15. *F. boothii*, 16. *F. graminearum* s.s.

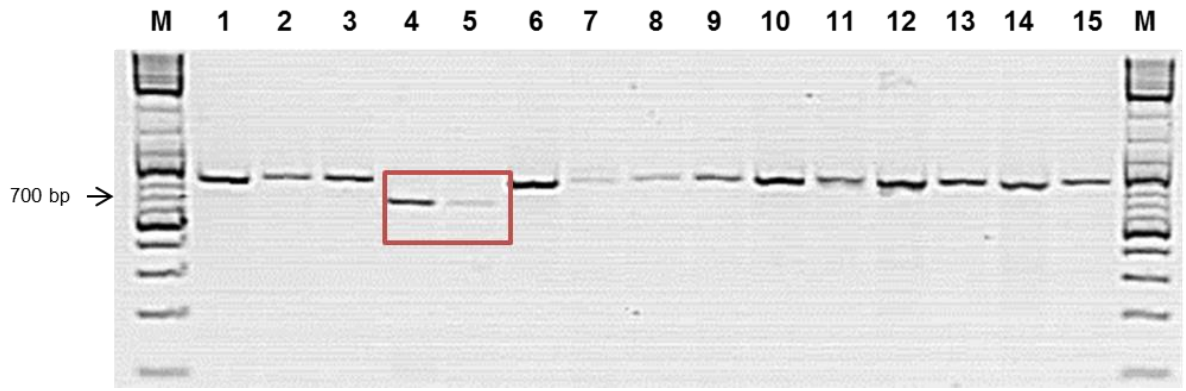

Figure 9. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Dra*I. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. graminearum* s.s., 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. meridionale*, 10. *F. meridionale*, 11. *F. meridionale*, 12 *F. boothii*, 13. *F. boothii*, 15. *F. boothii.*

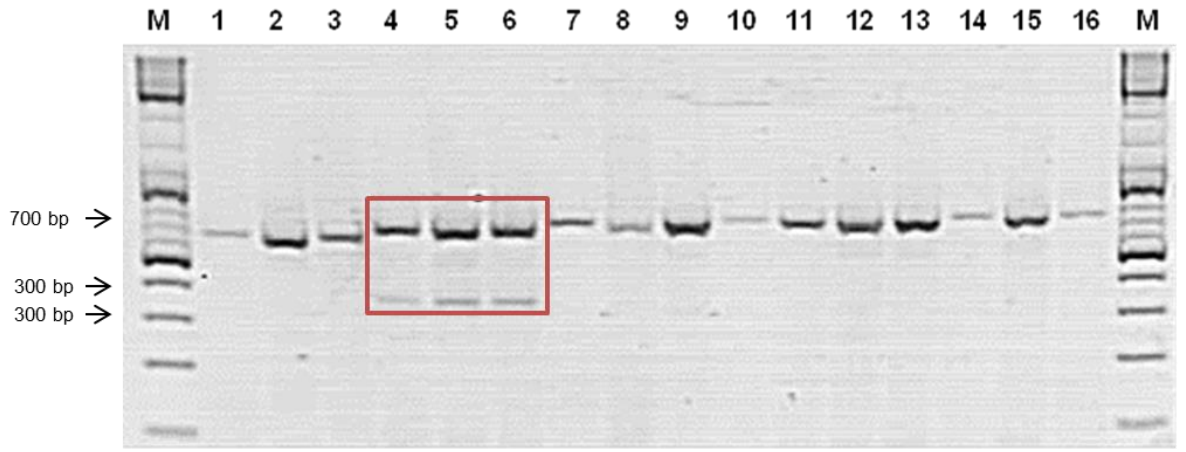

Figure 10. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Bse*GI. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. cortaderiae*, 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. graminearum* s.s., 10. *F. meridionale*, 11. *F. meridionale*, 12 *F. meridionale*, 13. *F. graminearum* s.s., 14. *F. graminearum* s.s., 15. *F. boothii,* 16. *F. boothii.*

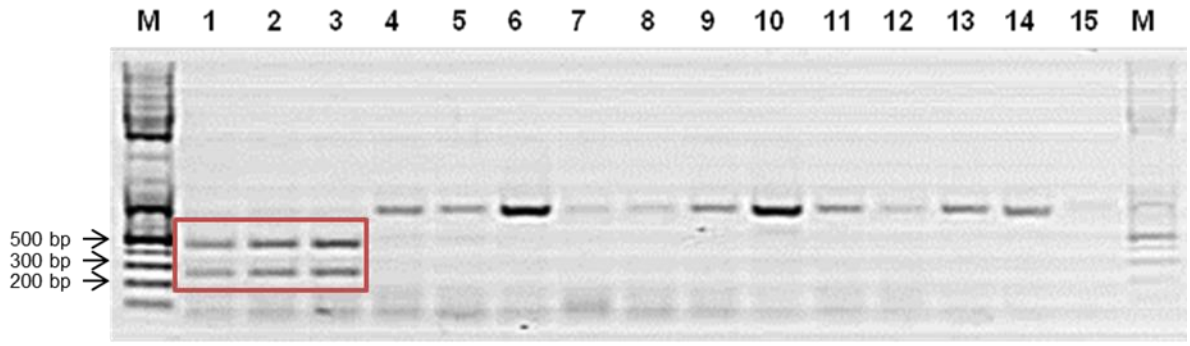

Figure 11. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Bfa*I. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. graminearum* s.s., 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. meridionale*, 10. *F. meridionale*, 11. *F. meridionale*, 12 *F. boothii*, 13. *F. boothii*, 14. *F. boothii*, 15. *F. graminearum* s.s.

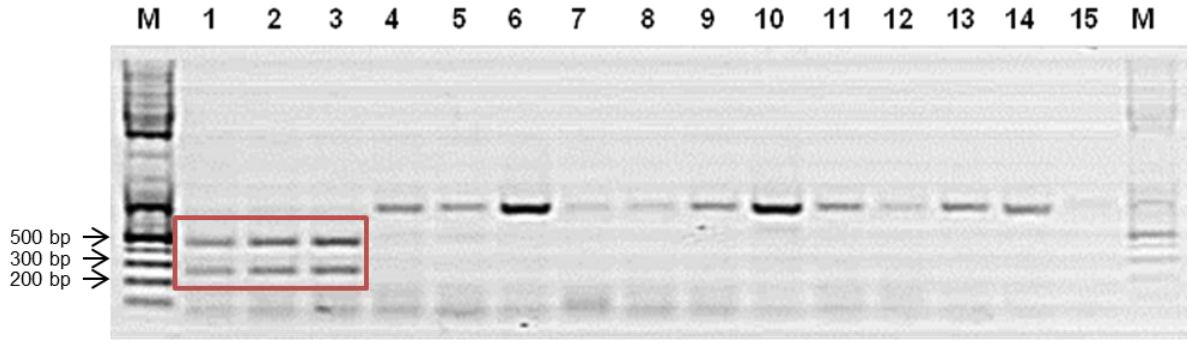

Figure 12. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Spe*I fast digest. M. The 100-bp GeneRuler DNA ladder, 1. *F. acaciae-mearnsii,* 2. *F. acaciae-mearnsii*, 3. *F. acaciae-mearnsii*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. graminearum* s.s., 7. *F. graminearum* s.s., 8. *F. graminearum* s.s., 9. *F. meridionale*, 10. *F. meridionale*, 11. *F. meridionale*, 12 *F. boothii*, 13. *F. boothii*, 15. *F. boothii.*

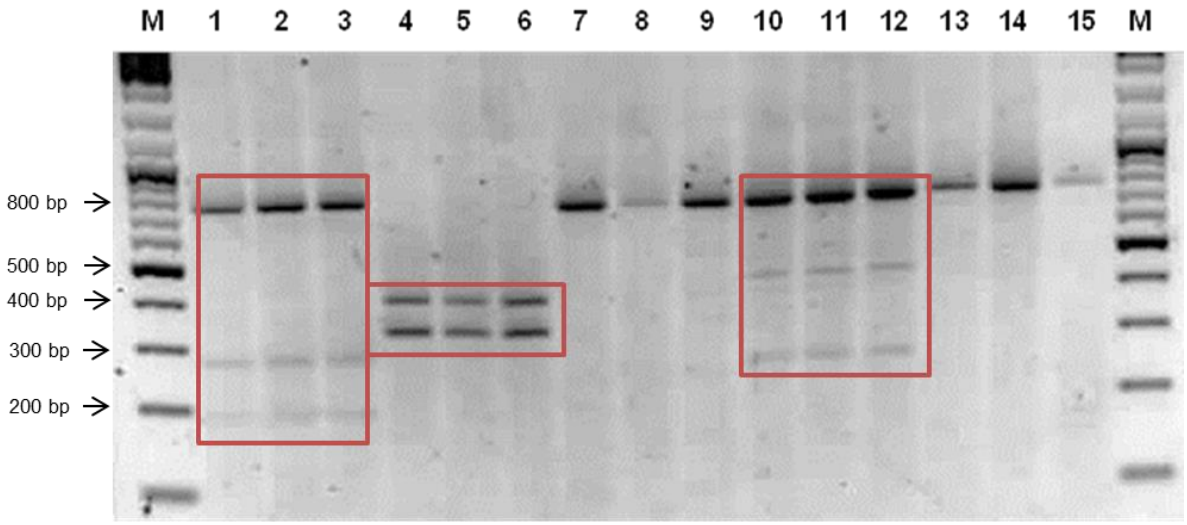

Figure 13. Double digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzymes *Bfa*I and *Bsa*HI. M. The 100-bp GeneRuler DNA ladder, 1. *F. cortaderiae,* 2. *F. cortaderiae*, 3. *F. cortaderiae*, 4. *F. graminearum* s.s., 5. *F. graminearum* s.s., 6. *F. graminearum* s.s., 7. *F. meridionale*, 8. *F. meridionale*, 9. *F. meridionale*, 10. *F. acaciae-mearnsii*, 11. *F. acaciae-mearnsii*, 12 *F. acaciae-mearnsii*, 13. *F. boothii*, 14. *F. boothii*, 15. *F. boothii*.

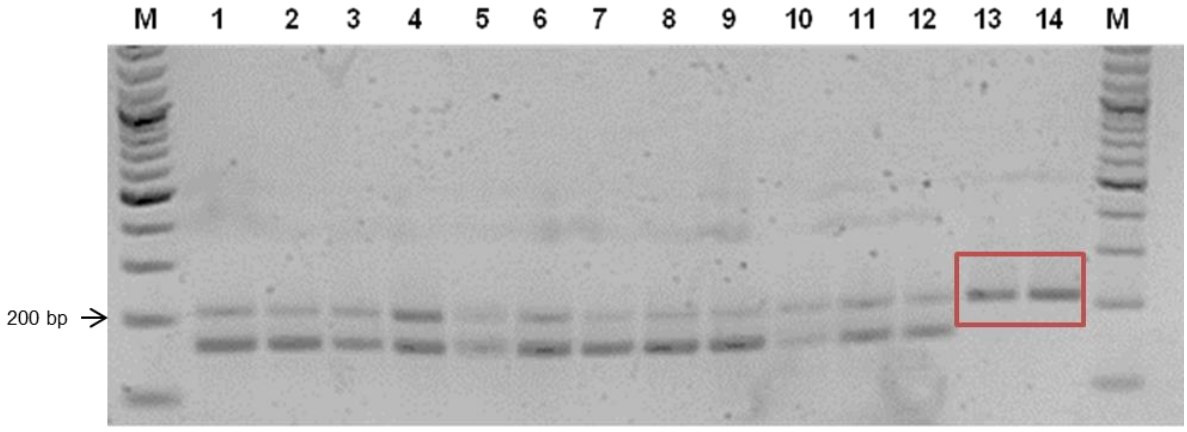

Figure 14. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Ear*I. M. The 100-bp GeneRuler DNA ladder, 1. *F. graminearum* s.s., 2. *F. graminearum* s.s., 3. *F. graminearum* s.s., 4. *F. meridionale*, 5. *F. meridionale*, 6. *F. meridionale*, 7. *F. acaciae-mearnsii*, 8. *F. acaciae-mearnsii*, 9. *F. acaciae-mearnsii*, 10. *F. cortaderiae*, 11. *F. cortaderiae*, 12. *F. cortaderiae*, 13. *F. boothii*, 14. *F. boothii.*

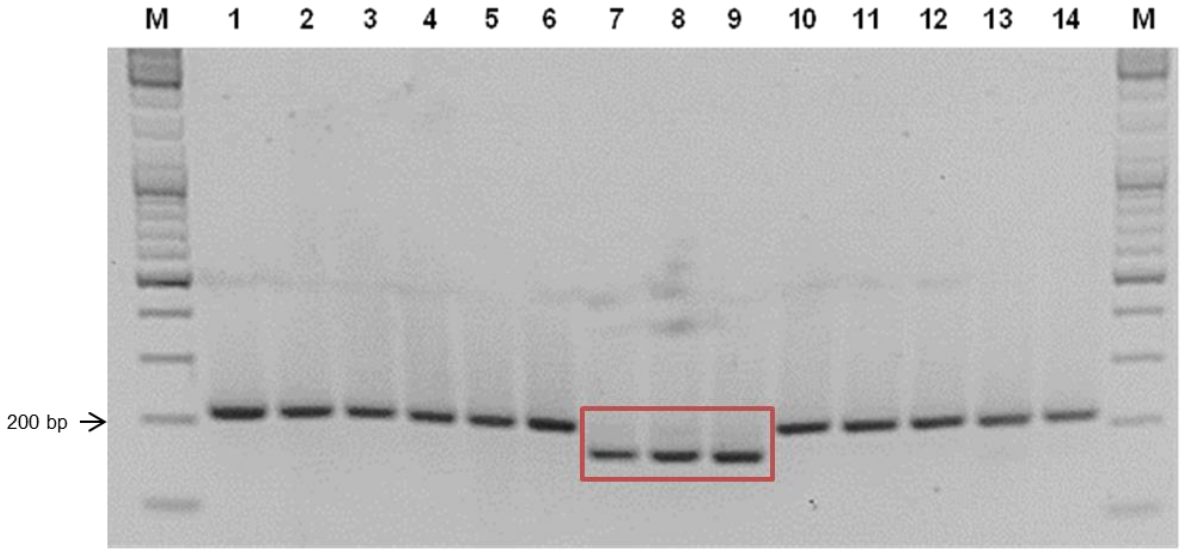

Figure 15. Digestion of the *translation elongation factor α-1* (*EF*-1α) region of *Fusarium* with the restriction enzyme *Tru*1I. M. The-100 bp GeneRuler DNA ladder, 1. *F. graminearum* s.s., 2. *F. graminearum* s.s., 3. *F. graminearum* s.s., 4. *F. meridionale*, 5. *F. meridionale*, 6. *F. meridionale*, 7. *F. acaciae-mearnsii*, 8. *F. acaciae-mearnsii*, 9. *F. acaciae-mearnsii*, 10. *F. cortaderiae*, 11. *F. cortaderiae*, 12. *F. cortaderiae*, 13. *F. boothii*, 14. *F. boothii.*

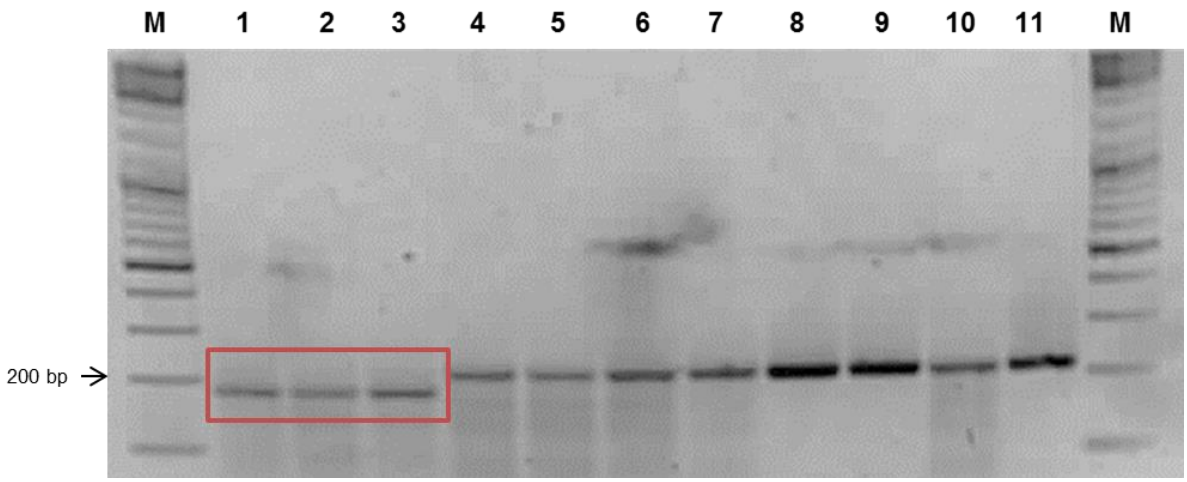

Figure 16. Digestion of the *histone* (*H*3) gene region of *Fusarium* with the restriction enzyme *Eco*RV. M. The 100 bp GeneRuler DNA ladder, 1. *F. graminearum* s.s., 2. *F. graminearum* s.s., 3. *F. graminearum* s.s., 4. *F. meridionale*, 5. *F. meridionale*, 6. *F. meridionale*, 7. *F. acaciae-mearnsii*, 8. *F. acaciae-mearnsii*, 9. *F. acaciae-mearnsii*, 10. *F. cortaderiae*, 11. *F. cortaderiae.*

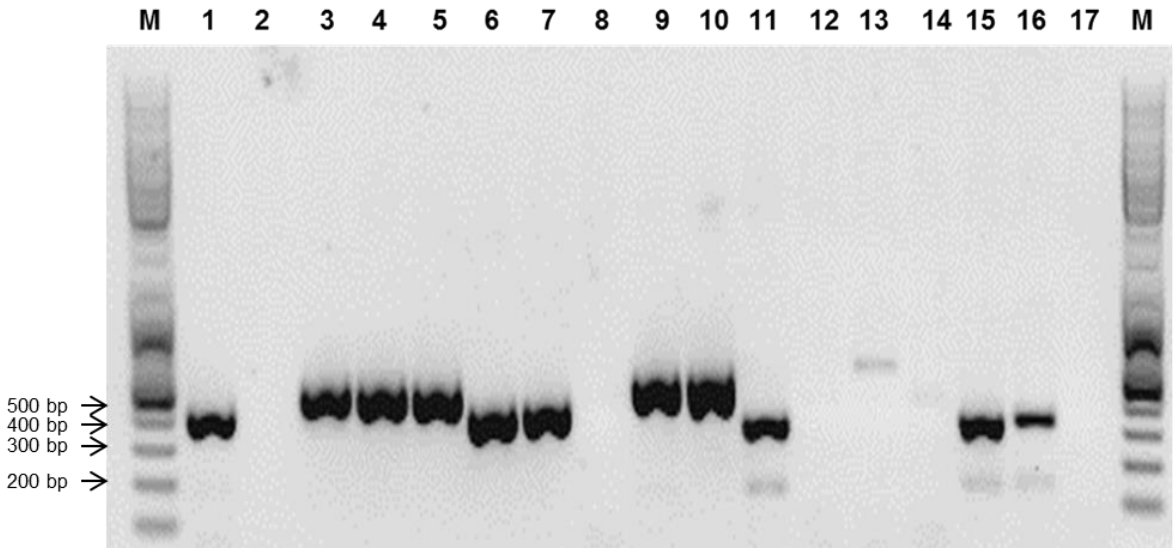

Figure 17. PCR products following the amplification of *Fusarium graminearum* species complex species with *F. graminearum*-specific primers. M. The 100-bp GeneRuler DNA ladder, 1. *F. graminearum* s.s., 2. *F. boothii*, 3. *F. cortaderiae*, 4. *F. cortaderiae*, 5. *F. cortaderiae*, 6. *F. graminearum* s.s., 7. *F. graminearum* s.s., 8. *F. boothii*, 9. *F. meridionale*, 10. *F. meridionale*, 11. *F. acaciae-mearnsii*, 12 *F. boothii*, 13. *F. boothii*, 14. *F. boothii*, 15. *F. acaciae-mearnsii*, 16. *F. acaciae-mearnsii,* 17. No template control.

CHAPTER 3

Phylogenetic species of the *Fusarium graminearum* species complex associated with wheat and maize grown in rotation in South Africa

ABSTRACT

Fusarium head blight (FHB) of wheat and Gibberella ear rot (GER) of maize is caused by a number of *Fusarium* species, including phylogenetic species of the *F. graminearum* species complex (FGSC). Infection of wheat and maize with FGSC species reduces crop yields and contaminates grain with mycotoxins. These mycotoxins include zearalenone (ZEA) and the Type B trichothecenes (TCTs); nivalenol (NIV), deoxynivalenol (DON) and their acetylated derivatives. The consumption of mycotoxin-contaminated grain has been associated with human and animal diseases. The aim of this study was to determine the distribution of FGSC species and their associated chemotypes from wheat and maize grown in rotational systems in South Africa for one cultivation seasons. Species identities of 779 isolates collected from 23 farms in seven districts were determined using molecular-based techniques. Chemotypes (15-ADON, 3-ADON or NIV) were determined by a multiplex PCR of the *acetyltransferase* (*Tri*3) gene. The *in vitro* production of NIV, fusaranone-X (FX), DON, 15-acetyl-deoxynivaleno (15-ADON) and ZEA by a subset of isolates was also determined. Of the wheat and maize isolates identified, 82.8 and 39.7% were *Fusarium graminearum* s.s., respectively. Only one isolate of *F. boothii* was isolated from wheat and none from maize. 15-ADON was the chemotype most frequently associated with wheat and maize isolates. Seven *Fusarium* isolates produced the NIV chemotype, but none of these isolates were FGSC species. The results of this study suggest that crop rotation with wheat and maize favours *F. graminearum* s.s., and may directly influence the FGSC species composition associated with these crops.

INTRODUCTION

The genus *Fusarium* consists of a number of phytopathogens that are globally distributed. Grain crops susceptible to these phytopathogens include wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and rice (*Oryza sativa* L.) (Van der Lee *et al*., 2015). Diseases caused to grain crops by *Fusarium* species include Fusarium head blight (FHB) of wheat, Gibberella ear rot (GER) and Fusarium ear rot (FER) of maize, as well as maize stem rot (Nicolaisen *et al*., 2009). The main *Fusarium* species associated with FHB and GER globally are *F. graminearum* sensu stricto (s.s.) Schwabe and *F. culmorum* (W.G. Smith) Saccardo (Bottalico and Perrone, 2002; Logrieco *et al*., 2002). FER of maize, however, is caused primarily by *F. verticillioides* (Sacc.) Nirenberg*, F. proliferatum* (Matsushima) Nirenberg and *F. subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (Logrieco *et al*., 2002),

Fusarium graminearum was initially considered a single fungal species based on its morphology. Recent advances made in the molecular identification of fungi have separated it into 16 phylogenetic species, collectively known as *F. graminearum* s.l. or the *F. graminearum* species complex (FGSC) (O'Donnell *et al*., 2000; 2004; Starkey *et al*., 2007; O'Donnell *et al*., 2008; Sarver *et al*., 2011). Species within the FGSC are widely distributed and have been reported in the United States, Canada, Europe and Africa (O'Donnell *et al*., 2000; 2004; Láday *et al*., 2004; Ramirez *et al*., 2007; Lee *et al*., 2009; Desjardins *et al*., 2011). The vast distribution of FGSC species is most likely due to the import and export of cereals, as well as changing environmental and climatic conditions (Qu *et al*., 2008). It has also been proposed that FGSC species are influenced by geographic location and that they display host preference (O'Donnell *et al*., 2000; Lee *et al*., 2009). Most of the surveys to study the distribution of FGSC species of grain crops have been conducted on wheat, with limited studies on maize (Boutigny *et al*., 2011).

Fusarium graminearum s.s. is the FGSC species most commonly associated with wheat. The fungus is primarily responsible for FHB in Europe (Waalwijk *et al*., 2003; Láday *et al.*, 2004), the USA (Starkey *et al*., 2007; Ward *et al*., 2008) and South Africa (Boutigny *et al*., 2011). In China, however, *F. asiaticum* O'Donnell, Aoki, Kistler *et* Geiser, is the dominant species associated with wheat, most likely because of its greater fitness under local environmental conditions (Qu *et al*., 2008; Gale *et al*., 2002). *Fusarium graminearum* s.s. is also commonly associated with maize, with few other FGSC species affecting the crop. Significant exceptions, however, do occur. In Nepal, *F. graminearum* s.s was not detected on GER diseased maize, however *F. asiaticum*, *F. meridionale* Aoki, Kistler, Geiser *et* O'Donnell and *F. boothii* O'Donnell, Aoki, Kistler *et* Geiser was the major causal agent of

GER of maize (Desjardins and Proctor, 2011). This finding has a significant implication in terms of disease management practices.

Diseases caused by FGSC species, including FHB and GER, result in poor grain quality and reduced grain yields. Additionally, grain of the affected crops is often contaminated with mycotoxins (McMullen *et al*., 1997; Windels, 2000; Bennett and Klich, 2003, Suga *et al*., 2008). The ingestion of mycotoxin-contaminated grain has been associated with acute health problems in humans as well as animals (D'Mello *et al*., 1999). The most frequently reported mycotoxins in cereals are the type B trichothecenes (TCT-B), deoxynivalenol (DON) and nivalenol (NIV). DON and NIV are produced by FGSC and other *Fusarium* species, including *F. culmorum*, along with their acetylated forms 15-ADON, 3-ADON and fusarenon-X (FX) (Bottalico, 1998). TCT-B mycotoxin contamination of crops can be predicted by determining the chemotype of the fungal species. Regional differences of the distribution of FGSC species TCTs have been observed. In Japan, the 15-ADON chemotype was mainly identified in the northern and the NIV chemotype in the central parts of the country (Yoshizawa and Jin, 1995). In South Africa the predominant chemotype identified from wheat, maize and barley was the 15-ADON chemotype (Boutigny *et al*., 2011), while the chemotype in wheat grain in Canada has shifted from 15-ADON to 3-ADON over time (Ward *et al*., 2008). Isolates within FGSC species can be of a specific chemotype, or they can host one of several different chemotypes (Wang *et al*., 2011). *Fusarium meridionale* isolates are all of the NIV chemotype (Goswami and Kistler, 2005; Starkey *et al*., 2007; O'Donnell *et al*., 2008; Ward *et al*., 2008), whereas *F. graminearum* s.s. and *F. asiaticum* O'Donnell, Aoki, Kistler *et* Geiser produce one of the three chemotypes; 15-ADON, 3-ADON and NIV (Wang *et al*., 2011).

In 2011, Boutigny *et al*. (2011) reported that 85.2% of FGSC isolates associated with FHB were *F. graminearum* s.s. and that 99.0% of the isolates from ears with GER were *F. boothii.* This suggested either a geographical separation of phylogenetic species, host-preference, or competition among wheat head and maize ear fungi. This information has important implications for crop rotation as a management strategy for FHB, GER and toxin accumulation of wheat and maize grain in South Africa, respectively. In the current study, the presence of phylogenetic species within the FGSC, and their mycotoxins, were therefore investigated in crop rotation systems in the country.

MATERIALS AND METHODS

Collection of plant material and isolation of fungi

Diseased wheat heads and maize ears displaying symptoms of FHB and GER, respectively, were collected from South African farms where the crops were grown in rotation. The wheat

and maize samples from the KwaZulu-Natal (KZN) and Northern Cape provinces were collected during the 2012/13 growing seasons from the same fields. Additionally, wheat and maize from the same fields were sampled from the Limpopo during the 2013/2014 growing seasons. Samples were only collected from farms where these crops were cultivated under irrigation. In total, 23 farms in seven districts of the Limpopo, KZN and Northern Cape provinces were surveyed during the 2012-2014 seasons. Four symptomatic and/or asymptomatic seeds were selected from each wheat head and maize ear. If there were few seeds displaying FHB or GER symptoms, asymptomatic seeds adjacent to the symptomatic seeds were selected. The seeds were surface-sterilised in 70% ethanol for 2 min and thoroughly rinsed in sterile distilled water. The washed kernels were then air dried in the laminar flow cabinet on sterile paper towel. Following drying, the kernels were placed onto potato dextrose agar (PDA) containing 40 mg L^{-1} streptomycin, and incubated for 2 days at 25°C. The fungal growth was sub-cultured onto ½ PDA without streptomycin, and incubated for 6 weeks at 25°C under 24-hr light. Putative *Fusarium* cultures were identified morphologically based on cultural growth characteristics and purified. They were then single-spored and stored at -80°C in the culture collection of the Department of Plant Pathology, Stellenbosch University, South Africa. Of the isolates from wheat and maize a total of 779 isolates were investigated for their species identity.

Species identification of putative *Fusarium* isolates

DNA extraction: A representative sample of isolates from wheat and maize were randomly selected for DNA isolation. Therefore, 507 and 272 isolates from wheat and maize respectively were selected. One isolate representing each of the FGSC species *F. graminearum* s.s., *F. cortaderiae* O'Donnell, T. Aoki, Kistler *et* Geiser, *F. acacia-mearnsii* O'Donnell, Aoki, Kistler *et* Geiser, *F.meridionale* and *F.boothii* were included as reference material. DNA was extracted from all fungal cultures grown on PDA using the Wizard® SV Genomic DNA Purification System (Promega, Wisconsin, U.S.A) according to the manufacturer's recommendations and the methodology described by Boutigny *et al*. (2011). Briefly, glass beads and 400 µL lysis buffer were added to the harvested mycelia collected in Eppendorf tubes and shaken for 10 min at a frequency of 30 with the Mixermill MM301 (Retsch GmbH, Mettmann, Germany). The samples were then incubated for 30 min at 65˚C, and centrifuged for 8 min at 14 000 revolutions per minute (rpm). The supernatant was transferred to a mini-column and collection tube assembly, and centrifuged for 3 min at 12 000 rpm. The liquid in the collection tube was discarded, 650 µL wash solution was added and the sample centrifuged for 1 min at 12 000 rpm. The washing step, including centrifugation, was repeated three times for a total of four washes. Following the final washing step the mini-column was centrifuged for 2 min at 12 000 rpm, and the mini-column

transferred to a new 1.5 mL micro-centrifuge tube. Nuclease-free water (75 µL) was added to the mini-column, incubated for 2 min, and centrifuged for 1 min at 12 000 rpm. This step was repeated twice, and the mini-column was thereafter discarded. RNase (1.2 µL) was added to each sample, vortexed and incubated for 10 min at room temperature. The DNA concentration was measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific, South Africa), and then stored at 4°C.

PCR - restriction fragment length polymorphism analysis: The identity of *Fusarium* isolates was determined by PCR - restriction fragment length polymorphism *(*RFLP), as described in Chapter 2. The *translation elongation factor α-1 (EF*-1α*)* gene region was amplified in a 25-µL reaction mixture containing 20 ng of DNA, 0.2 µM of primers EF1 and EF2 (O' Donnell *et al*., 1998; Geiser *et al*., 2004) (Table 1), 1x PCR buffer, 2.5 mM $MgCl_2$, 0.24 mM dNTP, 1.25µL of 20 mg mL^{-1} bovine serum albumin (BSA), and 0.3 U *Taq* DNA polymerase. The *histone* (*H*3) gene region was amplified in a mixture containing 20 ng of fungal DNA, 0.4 µM of each of the H3dStyI and H3R1 primers (O'Donnell *et al*., 2004; Suga *et al*., 2008) (Table 1), as well as 2x Ready Mix with Mg^{2+} (Kapa Biosystems, Cape Town, South Africa). The *EF*-1α and *H*3 PCRs were performed in a Thermocycler GeneAmp® PCR System 9700 (Applied biosystems, California, United States). Cycling conditions for the amplification of the *EF*-1α gene region were 5 min at 94°C; 35 cycles of 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C; before the final extension step of 7 min at 72°C. The amplification of the *H*3 gene region consisted of cycling conditions of 2 min at 95°C; followed by 35 cycles for 30 sec at 95°C, 45 sec at 56°C and 45 sec at 72°C; with a final extension step of 2 min at 72°C. The PCR products were loaded on a 1% agarose gel stained with GrGreen (Inqaba Biotec, Pretoria, South Africa), separated by gel electrophoresis and visualised under a UV transilluminator.

The restrictions enzymes *Bfa*I (New England Biolabs, Hitchin, United Kingdom) and *BsaH*I (New England Biolabs) were used in the double restriction digest of the *EF*-1α PCR product. The restriction digest reaction mixture included 10 U of each enzyme, 1x Cutsmart buffer and 15 µL of the *EF*-1α PCR product, in a total reaction volume of 50 µL. The reaction mixture was incubated for 1 hr at 37°C, followed by an inactivation step for 20 min at 80°C. The *H3* PCR product was digested with the restriction enzyme *Ear*I (New England Biolabs) according to the manufacturer's instructions. The restriction digest products were separated on a 2% agarose gel stained with GrGreen, the fragments separated by gel electrophoresis for 2 hr at 75 V, and the molecular size of each fragment determined using the 100-bp GeneRuler DNA ladder (Thermo Scientific).

Fusarium graminearum species-specific PCR: Fusarium isolates not identified by PCR-RFLP were subjected to a *F. graminearum* species-specific PCR assay (Chapter 2). The reaction mixture consisted of 20 ng of fungal DNA in a total reaction volume of 25 µL. The reaction mixture consisted of 1x buffer, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1.25 µL of 20 mg mL^{-1} BSA, 0.2 U taq DNA polymerase and 5 µM of each primer Fg16F/Fg16R (Table 1). The reaction mixture was subjected to thermal cycling conditions of 2 min at 94°C; followed by 35 cycles of 45 sec at 94°C, 30 sec at 58°C and 45 sec at 72°C; and a final extension step of 5 min at 72°C (Nicholson *et al*., 1998) in a thermocycler GeneAmp® PCR System 9700 (Applied biosystems). The PCR products were evaluated on a 1% agarose gel stained with GrGreen and separated by gel electrophoresis for 90 min at 75 V. The 100 bp GeneRuler DNA ladder was used to determine the molecular size of each fragment.

Sequencing of the intergenic spacer *gene region:* The *internal transcribed spacer* (*ITS*) gene region was sequenced for the identification of fungal isolates whose identity could not be determined by PCR-based techniques. The amplification of the ITS gene region was executed in a total reaction volume of 25 µl, comprised of 20 ng of fungal DNA, 0.2 µM of each primer ITS1/ITS4 (White *et al*. 1990), 1x buffer, 4 mM $MgCl_2$, 0.4 mM dNTPs, 1 µL of 20 mg mL^{-1} BSA and 1 U Taq DNA polymerase. The thermal parameters included an initial denaturing step for 5 min at 94°C; followed by 35 cycles of 94°C for 45 sec, 52°C for 30 sec and 72°C for 60 sec; with a final extension step at 72°C for 5 min. The PCR products were separated by gel electrophoresis on a 1% agarose gel stained with GrGreen and visualized under ultraviolet light. The PCR fragments of approximately 600 bp were purified using MSB® Spin PCRapace (Stratec Molecular, Berlin), according to the manufacturer's instructions. Sequencing was performed with the ABI 3730xl Genetic Analyser (Applied biosystems) by the Central Analytical Facility (CAF) at Stellenbosch University, and sequence products were generated using the ITS1 primer only. DNA sequences were analysed with Geneious (Version 5.3.3 Biomatters Ltd.) and compared to sequences available on the server of the US National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST/) and Fusarium-ID database (http://isolate.fusariumdb.org) using the basic local alignment search tool (BLAST).

Chemotyping of FGSC isolates

Multiplex PCR of the Tri3 region: The *Tri*3 gene regions of FGSC isolates collected from the farms and the reference cultures with known chemotypes were amplified using the primers 3CON, 3NA, 3D3A and 3D15A (Ward *et al.*, 2002) (Table 1). The reference cultures INRA 156, INRA 233 and INRA 91 represented the TCT chemotypes 15-ADON/DON, 3-ADON/DON and NIV/FX, respectively, and were provided by French National Institute for

Agricultural Research, Villenave d'Ornon, France. The amplification was performed using 10 ng μL^{-1} of fungal DNA in a total reaction mixture of 25 µL. The reaction mixture consisted of 0.4 µM of each of the four primers (O'Donnell *et al*., 2004; Suga *et al*., 2008) (Table 1), as well as 2x Ready Mix with Mg^{2+} (Kapa Biosystems). The *Tri*3 gene was amplified in a Thermocycler GeneAmp® PCR System 9700 (Applied Biosystems). Thermal cycling conditions included 2 min at 94°C; followed by 35 cycles of annealing for 30 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C; with a final extension step of 5 min at 72°C. The PCR products were separated by gel electrophoresis on a 1% agarose gel, stained with GrGreen and visualised under a UV transilluminator. The amplicon size was determined with a 100-bp GeneRuler DNA ladder (Thermo Scientific). Amplicons of 610 bp, 243 bp and 840 bp represented the 15-ADON, 3-ADON and NIV chemotypes, respectively (Ward *et al*., 2008).

In vitro *mycotoxin production*: Five positive controls and one negative control were included in the evaluation of the *in vitro* mycotoxin production of 18 FGSC isolates with pre-determined chemotypes. The isolates were grown on PDA for 4 days at 25°C. An agar plug from the developing colonies was transferred to 500-mL Erlenmeyer flasks containing 25 g rice that were soaked overnight in 15 mL deionized water and autoclaved. The flasks were then incubated for 42 days under 24 hour light at 25°C. Mycotoxins were extracted by adding 100 mL of a 70% methanol extraction solvent to each sample, mixing them thoroughly with a spatula, and shaking them for 60 min at 200 rpm at 25°C. Approximately 40 mL of the mixtures were filtered through double-layered sterile cheese cloth, and centrifuged for 10 min at 4 000 rpm at 4°C. The supernatants (20 mL) were transferred into new tubes and centrifuged for 10 min at 4 000 rpm at 4°C. The supernatants were filter-sterilised into a 2-mL micro-centrifuge tube through 0.20-µm RC filters, and incubated overnight at 4°C. The samples were then centrifuged for 10 min at 14 000 rpm, and 1.8 mL of the supernatant transferred to glass vials for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis at CAF, Stellenbosch University. The LC-MS/MS analysis was performed on a Waters Xevo TQ MS with Waters Accuity UPLC (Waters, Massachusetts, United States).

Statistical analysis

Analysis of variance (ANOVA) employing the Generalized linear model (GLM) procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA) was performed for mycotoxin (NIV, FX, DON, 15-ADON and ZEA) contamination.

Weather data collection

The weather data for the respective districts where FHB wheat and GER maize samples were collected was provided by the Institute for Soil Climate and Water in Pretoria, South Africa. Daily maximum temperature (Tx), daily minimum temperature (Tn), total rainfall (Rain), daily maximum relative humidity (RHx) and daily minimum relative humidity (RHn) were obtained. Weather data for the Northern Cape and KZN provinces were collected during the 2012 wheat season, whereas weather data for the Limpopo province was collected during the 2013 wheat session (Table 6). For wheat, the data were collected in September and October, as these months coincided with anthesis when heads are most susceptible to infection (Sutton, 1982). For maize, weather data was collected in February and March during the soft dough stage, which is when the ears are most susceptible to infection. Weather data for the Northern Cape and KZN provinces were collected during the 2013 maize season, and for the Limpopo province during the 2014 maize season.

RESULTS

Species identification

A total of 507 isolates from wheat and 272 isolates from maize were identified as *Fusarium* species based on cultural morphology. This comprised 36.9 and 80.2% of the fungal cultures obtained from wheat and maize, respectively (Table 2). Of the FGSC species known to occur in South Africa, *F. graminearum* s.s. and *F. boothii* were isolated from wheat, and only *F. graminearum* s.s. from maize. The wheat isolates were identified as *F. graminearum* s.s. (82.8%) and as *F. boothii* (0.2%), and *F. graminearum* s.s. represented 39.7% of the fungal cultures originally identified as *Fusarium* species from maize (Table 3). In addition, 14.6 and 14% of wheat and maize isolates, respectively, were unidentified FGSC species. Other *Fusarium* species found in maize included *F. verticillioides* (16.9%), *F. proliferatum* (9.2%), *F. culmorum* (2.2%), *F. chlamydosporum* Wollenweber & Reinking. (4%), *F. oxysporum* Schlechtendahl emend. Snyder & Hansen (4%), *F. polyphialidicum* Marasas, Nelson, Toussoun & van Wyk (4%), *F. temperatum* Scauflaire *et* F. Munaut, (4%) and an unknown *Fusarium* species (4%) (Fig. 1). A single isolate of *F. culmorum* was also associated with wheat. One isolate of an *Aspergillus* species and one of *Epicoccum sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley were found on maize, while one isolate of a *Glomerella* species was associated with wheat. A total of 52 (6.7%) isolates of the collection of 779 isolates could not be identified (Table 3).

Fusarium graminearum s.s. was the most commonly isolated FGSC species from both wheat and maize in the three provinces sampled. Of the 141 isolates obtained from wheat in Douglas, 118 isolates were identified as *F. graminearum* s.s., 22 isolates as *F.*

graminearum s.l. and one isolate was identified as another fungal species (Fig. 2). Additionally 74 isolates from maize cultivated in Douglas were identified, of which 20 isolates were identified as *F. graminearum* s.s., 12 isolates as *F. graminearum* s.l., 18 isolates as other *Fusarium* spp. and 24 isolates were unidentified. Isolates were collected from Prieska of which 61 isolates were from wheat and 57 isolates were from maize. The fungal species identified from wheat included *Fusarium graminearum* s.s. (33 isolates), *F. graminearum* s.l. (22) and 6 isolates were unidentified. Of the 57 isolates from maize obtained from Prieska, 26 isolates were identified as *F. graminearum* s.s., 12 isolates as *F. graminearum* s.l., 7 isolates as other Fusarium species, 1 isolate as other fungal species and 11 isolates were unidentified. 65 and 52 isolates from wheat and maize, respectively, were collected from Jacobsdal. Of the isolates from wheat 41 isolates were identified as *F. graminearum* s.s. and 24 isolates as *F. graminearum* s.l. Furthermore the isolates from maize were identified as *F. graminearum* s.s. (33 isolates), *F. graminearum* s.l. (5 isolates), other *Fusarium* species (12 isolates), other fungal species (1 isolates) and one isolate was unidentified. Of the isolates from wheat, collected from Winterton, *F. graminearum* s.s. (21 isolates) and *F. boothii* (1 isolates) were identified. The maize isolates from Winterton were identified as *F. graminearum* s.l. (4 isolates), other *Fusarium* species (6 isolates) and one isolate was unidentified. The isolates from wheat obtained from Groblersdal were identified as *F. graminearum* s.s. (59 isolates), *F. graminearum* s.l. (1 isolates) and one isolate was not unidentified. Of the Groblersdal maize isolates *F. graminearum* s.s. (10 isolates), *F. graminearum* s.l. (2 isolates) and other *Fusarium* species (21 isolates) were identified. *F. graminearum* s.s. (127 isolates), *F. graminearum* s.l. (5 isolates) and two unidentified species was identified from wheat obtained from Koedoeskop. Furthermore the species identified from maize planted in Koedoeskop were identified as F*. graminearum* s.s. (18 isolates), *F. graminearum* s.l. (1 isolates) and other *Fusarium* species (11 isolates) (Fig. 2).

Chemotype of FGSC isolates

Multiplex PCR of the Tri3 region: Of the 779 isolates evaluated, 740 produced clear identities and were chemotyped. Chemotyping of 740 FGSC and other *Fusarium* species showed that 73.0% of the isolates produced a TCT chemotype product, whereas 27.0% of the isolates did not produce any chemotype. The TCT chemotypes included 15-ADON and NIV, but not 3-ADON. The 15-ADON chemotype was most abundant, representing 80.8% of the wheat isolates, only one isolate (0.2%) was of the NIV chemotype (Table 4). Ninety-five isolates (19.0%) from wheat did not produce any TCT chemotype amplification product. Of the maize isolates, 53.9% produced the 15-ADON chemotype and 2.5% the NIV chemotype (Table 4). However, 43.6% did not produce a chemotype (Table 4).

In vitro *mycotoxin production*: All the isolates tested for mycotoxin production *in vitro*, except two, produced either DON and its acetylated form or NIV and its acetylated form, as well as ZEA, on rice media (Table 5). The DON controls M0002, 2.945 and 2.574 produced both DON and 15-ADON, although 15-ADON concentrations were low (0.04 to 0.13 parts per million [ppm]). The NIV controls produced NIV and FX toxin *in vitro*. Nine wheat and maize isolates collected in South Africa produced DON at concentrations ranging from 0-8.56 ppm (Table 5). W12.377, collected from wheat, produced the highest DON concentration of 8.56 ppm, which was significantly more than that produced by the DON controls and the other South African isolates. Of the isolates collected from maize, M14.1 produced the highest DON concentration (1.77 ppm), which was significantly more than the other isolates collected from maize (Table 5). ADON toxin was produced at concentrations of 0-0.38 ppm (Table 5). Isolate W12.377 produced the most (0.38 ppm) and W13.93 (0.15 ppm) the second most 15-ADON, which was significantly more than that produced by the other 15-ADON producers (Table 5). Four isolates with a 15-ADON chemotype; W13.578, M13.94, M13.80 and M13.214; did not produce detectable levels of DON and 15-ADON. No 3-ADON was produced by any of the isolates.

NIV production ranged from 0-8.28 ppm, and FX production from 0-0.75 ppm. Isolate M14.31 from maize was the highest producer of NIV and FX, and produced significantly more than any of the other isolates (Table 5). None of the isolates collected from wheat produced detectable levels of FX (Table 5). Fifteen of the 18 isolates evaluated produced ZEA at concentrations of 0-50.22 ppm (Table 5). Isolate W13.400 produced most ZEA, but not significantly more than W12.377 (45.09 ppm), M14.54 (47.25 ppm) and the positive control 2.945 (46.70 ppm). Detectable levels of ZEA were not produced by isolates M13.214, M14.31 and M14.26; but it was found in the negative control.

Weather data

During September and October, the average daily minimum temperature was lowest in the three Northern Cape districts during 2012 (3.2-9.5°C), and the average maximum temperature highest in KZN and Limpopo in 2013 (30.0-31.0°C). Although all fields were irrigated, the average total rainfall varied substantially among districts. This resulted in a higher maximum relative humidity in KZN (90.4-92.0%) compared to the Limpopo (67.6-90.2%) and Northern Cape provinces (73.5-89.4%) (Table 6). Average daily minimum temperature ranged between 12.2 and 18.6°C in maize fields in February and March, with the highest temperatures recorded in the Limpopo province (17.6-18.6°C) during the 2014 planting season. The average daily maximum temperatures, however, were highest in the Northern Cape Province in 2013, where it ranged from 33.3-37.6°C. Daily maximum relative

humidity was above 90% in all districts, except for Prieska in 2013 and Koedoeskop in February 2014 (Table 7).

DISCUSSION

In this study, *F. graminearum* s.s. was identified as the most common FGSC species associated with wheat and maize grain grown in rotation. While this finding was not entirely surprising considering the wide distribution of *F. graminearum* s.s. globally (O'Donnell *et al*., 2000; 2004; Láday *et al*., 2004; Monds *et al*., 2005; Ramirez *et al*., 2007; Suga *et al*., 2008; Desjardins and Proctor, 2011), its abundance on maize was in contrast to the study conducted by Boutigny *et al*. (2011), who reported *F. boothii* to be the only FGSC species associated with South African maize grain. Qiu & Shi (2014) only identified *F. graminearum* s.s on maize. The current investigation, thus, provided new insights into FGSC dynamics under different production systems, geographic locations and climatic conditions.

FGSC species distribution on grain crops is markedly influenced by cropping system and geographical location (Zhang *et al*., 2012). A study by Desjardins and Proctor (2011) proposed that a rice and maize rotational cropping system provided an environment beneficial for the migration of *F. asiaticum* from rice to maize. The FGSC species present on South African maize in the current study (*F. graminearum* s.s.), when compared to the FGSC species distribution reported by Boutigny *et al*. (2011) (*F. boothii*), could be attributed to the rotation of wheat and maize under irrigation in combination with other variables including climate conditions. When *F. boothii* on maize ears were identified by Boutigny *et al*. (2011) they were collected from monoculture fields cultivated under dryland conditions, and in districts different from the ones surveyed in the current study. The absence of *F. cortaderiae*, *F. acacia-mearnsii*, *F. meridionale*, and *F. brasilicum* on wheat in South Africa; previously reported by Boutigny *et al*. (2011); further illustrates the importance of geographic location on FGSC species composition. All of these FGSC species were collected by Boutigny *et al*. (2011) at localities not surveyed in the current study.

Fusarium graminearum s.s. was the dominant FGSC species responsible for FHB of wheat grown under irrigation in South Africa (Scott *et al*., 1988; Kriel and Pretorius, 2008; Boutigny *et al*., 2011). Crop rotation of wheat and maize, and reduced tillage practices, allows *F. graminearum* s.s. to overwinter as perithecia on the previous season's stubble (Osborne and Stein, 2007; Kriel and Pretorius, 2008). These perithecia then release ascospores that can serve as primary inoculum and cause disease on susceptible hosts in the new cropping season. This could explain the prevalence of *F. graminearum* s.s. on maize produced under irrigation when it was rotated with wheat. This study suggests that it is not adviable to rotate wheat and maize. Therefore rotation of wheat and maize under

irrigation in South Africa, should be limited or prevented where possible to effectively manage FHB, GER, and their associated mycotoxins in the country. However, rotation with wheat and maize as well as non crop rotation should be further investigated and compared to determine the impact of crop rotational systems on the presence of FGSC species.

Irrigation, in combination with environmental factors such as heat and humidity, provides an environment conducive for FHB development (Wiese, 1987; Bai and Shaner, 1994; Markell and Francl, 2003). In KZN, where the highest maximum temperatures were recorded, the FGSC composition was dominated by *F. graminearum* s.s. However, the unknown *F. graminearum* s.l. isolates comprised 25% of the total isolates collected from wheat in the Northern Cape province, where average minimum temperatures were the lowest. These findings suggest that minimum and maximum temperatures could potentially influence FGSC diversity in certain locations, although more research is required to substantiate this observation. Relative humidity appeared to have less of an influence on the composition of *Fusarium* species on maize. Sufficient moisture was available through irrigation for infection to occur, even though relative humidity differed among districts. Persistent wet weather and warm conditions during the susceptible stage of maize also allows for the development of GER (Sutton, 1982). In the current study, however, the high average maximum temperatures and low average humidity in the Northern Cape province did not appear to substantially influence the Fusarium species composition on maize ears. The most likely explanation for this is that free moisture and temperature regimes in all districts surveyed were sufficient for infection of FGSC and other *Fusarium* species to occur,

A few FGSC isolates from maize and wheat were tentatively identified as *F. graminearum* s.l. by DNA sequencing of the ITS gene region. These isolates' identities could also not be resolved with PCR-RFLPs and a *F. graminearum* species-specific PCR, which distinguished among the five FGSC species already present in South Africa. Due to the failure of amplification of these unknown fungal isolates it was assumed these isolates were not *Fusarium* spp. therefore the ITS gene region was amplified and sequenced. The *F. graminearum* s.s. isolates could, therefore, potentially represent FGSC species not previously reported in South Africa, and their identities should thus be confirmed by multi-locus genotyping. Additionally the ITS gene region serves as a preliminary identification method, The *EF*-1α or RNA polymerase gene regions should be sequenced for accurate identification of *Fusarium* species. Accurate identified of FGSC species is not only important for understanding the diversity of the fungal complex in South Africa, but also to predict their potential mycotoxin contamination risks.

Non-FGSC species were associated with wheat and maize, with a greater diversity found on maize. *Fusarium culmorum*, a *Fusarium* species commonly associated with FHB on wheat in Europe, was identified on both wheat and maize in South Africa. *Fusarium*

culmorum is often isolated from environments with temperate to cool climates, where it sometimes also colonises maize (Logrieco *et al*., 2002). The non-FGSC species most commonly found on maize were *F. verticillioides* and *F. proliferatum.* These pathogens cause FER, and they have been reported to co-occur on maize (Ross *et al*., 1990). *Fusarium verticillioides* is known as the most dominant pathogen on maize grain in South Africa (Marasas *et al*., 1979; Marasas, 1995; Boutigny *et al*., 2012), while *F. proliferatum* is seldom isolated from local maize grain (Boutigny *et al*., 2012). A study by Ncube *et al*. (2011), however, reported *F. proliferatum* on maize grain produced by subsistence farmers in South Africa. *Fusarium proliferatum* has previously been reported on wheat grain (Amato *et al*., 2015), but not *F. verticillioides*. Both are producers of fumonisins with potential health implications to humans and animals (van der Lee *et al*., 2015). It is thus important that neither *F. verticillioides* or *F. proliferatum* emigrate from maize to wheat under irrigation in a crop rotational system.

Mycotoxigenic *Fusarium* species of the 15-ADON and NIV chemotypes were identified in this study. The 15-ADON chemotype, produced by FGSC species *F.graminearum s.s* and *F. boothii*, was found to be the most dominant chemotype in South Africa, as previously reported (Boutigny *et al*., 2011). The same chemotype was also most abundant in Argentina, Brazil, China, Netherlands, the USA and the United Kingdom (Waalwijk *et al*., 2003; Jennings *et al*., 2004; Ji *et al*., 2007; Scoz *et al*., 2009; Reynoso *et al*., 2011). *Fusarium graminearum* s.s., however, also has the potential to produce 3-ADON and NIV (Wang *et al*., 2011). In the current study, wheat and maize were also colonised by the NIV-producing *F. culmorum*. DON-producing isolates are more virulent on wheat than NIV-producing isolates (Desjardins *et al*., 2004). However, studies have reported that grain contaminated with DON is less toxic to human and animals than grain contaminated with NIV (Ryu *et al*., 1988). Both DON and NIV are subjected to regulations in more than 70 countries (Haumann, 1995; Van Egmond *et al*., 2007).

The presence of *F. culmorum* on wheat and maize, and its demonstrated ability to produce NIV *in vitro*, may have negative implications for South African grain production (Forsell and Pestka, 1985). Boutigny *et al*. (2011), however, suggested that NIV contamination of wheat, maize and barley grain in South Africa would be uncommon because of the preference of *F. culmorum* for cooler climates. More concerning could be the ability of FGSC species found in South Africa to produce ZEA. ZEA has several negative effects on livestock. The consumption of ZEA-contaminated feed has been linked with reproductive problems in swine (Nelson *et al*., 1993). The FGSC isolates collected in this study were all able to produce ZEA in abundance *in vitro*.

In conclusion, this study identified two FGSC species (*F. graminearum* s.s. and *F. boothii*) on wheat and a single FGSC specie (*F. graminearum* s.s.) was identified on maize.

No other FGSC species, previously reported on South African wheat and maize, was identified within this study. Several isolates were identified a *F. graminearum* s.l. The only chemotype associated with the FGSC species on both wheat and maize in this study was the 15-ADON chemotype. Future research should compare and evaluate the diversity of the FGSC species on wheat and maize crop rotational systems to the FGSC species identified on wheat and maize not grown in a rotational system. The isolates identified as *F. graminearum* s.l. by DNA sequencing should be subjected to futher identification of the specific FGSC species.

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Table 1. Gene regions, primer names and sequences used in the determination of species identity and chemotype of the fungal isolates collected from fields that roate wheat and maize in South Africa.

Target	Primer name	Primer sequence (5'---3')	Reference
EF-1α	EF1	ATGGGTAAGGA(A/G)GACAAGAC	O' Donnell *et al*. (1998); Geiser *et al*. (2004)
	EF2	GGA(G/A)GTACCAGT(G/C)ATCATGTT	O' Donnell *et al*. (1998); Geiser *et al*. (2004)
*H*3	H3dStyI	AGCATCACCYGAACATCGCATCATCCCATG	Suga *et al*. (2008)
	H3R1	TTGGACTGGATRGTAACACGC	O'Donnell *et al*. (2004)
F. graminearum	Fg16F	CTCCGGATATGTTGCGTCAA	Nicholson *et al*. (1998)
	Fg16R	GGTAGGTATCCGACATGGCAA	Nicholson *et al*. (1998)
ITS	ITS1	TCCGTAGGTGAACCTGCG	White *et al*. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
*Tri*3	3CON	TGGCAAAGACTGGTTCAC	Ward *et al*. (2008)
	3NA	GTGCACAGAATATACGAGC	Ward *et al*. (2008)
	3D15A	ACTGACCCAAGCTGCCATC	Ward *et al*. (2008)
	3D3A	CGCATTGGCTAACACATG	Ward *et al*. (2008)

Table 2. The number of fungal isolates obtained from wheat with Fusarium head blight symptoms and maize with Gibberella ear rot symptoms in South Africa.

Host	Year[1, 2]			Total[1]
	2012	2013	2014	
Wheat	289 (760)	218 (613)	-	507 (1 373)
Maize	-	209 (256)	63 (83)	272 (339)
Total	289	427	63	779 (1 712)

[1]Numbers represent the *Fusarium*-like isolates collected in wheat and maize fields, and those in brackets represent the total number of fungal isolates collected

[2] '-' indicates that no samples were collected in these seasons

Table 3. The representation of *Fusarium graminearum* species complex (FGSC) species collected from wheat and maize in South Africa.

Species	Host	
	Wheat (%)[1]	Maize (%)[2]
F. graminearum	82.8	39.7
F. boothii	0.2	0.0
F. cortaderiae	0.0	0.0
F. acacia-mearnsii	0.0	0.0
F. meridionale	0.0	0.0
F. brasilicum	0.0	0.0
F. graminearum s.l.	14.6	14.0
Non-FGSC species	0.4	30.9
Unidentified species	2.0	15.4
Total	507	272

[1] Percentage of isolates representing species identity from the total wheat evaluated

[2] Percentage of isolates representing species identity from the total maize evaluated

Table 4. Chemotypes of *Fusarium graminearum* species complex isolates collected from wheat and maize in South Africa.

Host	Chemotype (%)				Total
	15-ADON[1]	3-ADON[2]	NIV[3]	No Chemotype	
Wheat	80.8	0.0	0.2	19.0	499
Maize	53.9	0.0	2.5	43.6	241

[1]15-acetyl-deoxynivalenol (15-ADON) chemotype produced by *F. graminearum* s.s. and *F. boothii*

[2]3-acetyl-deoxynivalenol (3-ADON)

[3] Nivalenol (NIV) produced by *F. culmorum*

Table 5. Mean concentrations of deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), fusarenon-X (FX) and zearalenone (ZEA) produced *in vitro* by *Fusarium graminearum* species complex isolates collected from maize and wheat in South Africa.

[1]Isolate number	Molecular Chemotype	Species identity	Toxin produced *in vitro* (ppm)[4]				
			DON	15-ADON	NIV	FX	ZEA
W13.93	15-ADON	*F. graminearum* s.s.	3.10 d	0.15 b	0.00 c	0.00 c	34.32 de
W13.602	15-ADON	*F. graminearum* s.s.	1.49 ef	0.00 e	0.00 c	0.00 c	38.26 cd
W13.578	15-ADON	*F. graminearum* s.s.	0.00 h	0.00 e	0.00 c	0.00 c	0.35 g
W13.400	15-ADON	*F. graminearum* s.s.	2.10 e	0.04 c-e	0.04 c	0.00 c	50.22 a
W12.377	15-ADON	*F. graminearum* s.s.	8.56 a	0.38 a	0.12 c	0.00 c	45.09 abc
W12.311	15-ADON	*F. graminearum* s.s.	0.45 gh	0.00 e	0.00 c	0.00 c	14.56 f
W12.279	15-ADON	*F. graminearum* s.s.	5.08 b	0.11 b-d	0.05 c	0.00 c	40.08 bcd
M14.7	15-ADON	*F. graminearum* s.s.	1.32 ef	0.03 de	0.00 c	0.00 c	35.89 de
M14.54	15-ADON	*F. graminearum* s.s.	0.91 fg	0.04 c-e	0.00 c	0.00 c	47.25 ab
M14.1	15-ADON	*F. graminearum* s.s.	1.77 e	0.02 de	0.04 c	0.00 c	32.83 de
M13.94	15-ADON	*F. graminearum* s.s.	0.00 h	0.00 e	0.00 c	0.00 c	0.01 g
M13.80	15-ADON	*F. graminearum* s.s.	0.00 h	0.00 e	0.00 c	0.00 c	0.29 g
M13.214	15-ADON	*F. graminearum* s.s.	0.00 h	0.00 e	0.00 c	0.00 c	0.00g
M14.31	NIV	*F. culmorum*	0.00 h	0.00 e	8.28 a	0.75 a	0.00g
M14.29	NIV	*F. culmorum*	0.00 h	0.00 e	0.87 bc	0.29 b	0.35 g
M14.26	NIV	*F. culmorum*	0.00 h	0.00 e	0.00 c	0.00 c	0.00g
M14.19	NIV	*F. culmorum*	0.00 h	0.00 e	0.21 c	0.09 c	0.10 g
M14.15	NIV	*F. culmorum*	0.00 h	0.00 e	0.29 c	0.09 c	0.19 g
2.945	[2]3-ADON	*F. brasillicum*	1.87 e	0.04 c-e	0.00 c	0.00 c	46.70 ab
M0002	[2]DON	*F. boothii*	3.21 d	0.05 c-e	0.04 c	0.00 c	7.54 fg
2.574	[2]DON	*F. graminearum* s.s.	4.03 c	0.13 bc	0.00 c	0.00 c	30.12 e
2.905	[2]NIV	*F. meridionale*	0.00 h	0.00 e	0.17 c	0.11 c	15.30 f
2.889	[2]NIV	*F. acaciae-mearnsii*	0.00 h	0.00 e	1.65 b	0.63 a	1.79 g
[3]NC			0.00 h	0.00 e	0.00 c	0.00 c	0.15 g

[1]W: Wheat, M: Maize
[2]Positive control
[3]Negative control
[4] Values in colunms followed by the same letter do not differ significantly (P<0.05)

Table 6. Weather data collected in six wheat-growing districts in South Africa during September and October of the 2012 and 2013 production seasons.

Crop	Province	District	Year	Month	Average Tn (°C)[1]	Average Tx (°C)[2]	Average RHn (%)[3]	Average RHx (%)[4]	Average Rain (mm)
Wheat	Northern Cape	Douglas	2012	September	3.2	24.9	17.9	87.3	6.2
				October	7.2	28.4	19.7	89.4	0.5
		Prieska	2012	September	3.8	25.2	14.1	73.5	0.0
				October	8.6	28.4	17.2	75.6	0.9
	KwaZulu-Natal	Jacobsdal	2012	September	5.0	24.4	16.1	79.2	0.1
				October	9.5	28.2	17.0	80.9	0.1
		Winterton	2012	September	7.6	24.3	30.4	90.4	4.6
				October	11.2	26.5	36.6	92.0	4.6
	Limpopo	Groblersdal	2013	September	11.3	30.5	21.3	74.1	0.1
				October	13.4	30.0	30.6	90.2	4.5
		Koedoeskop	2013	September	11.0	30.3	16.1	67.6	0.2
				October	13.6	31.0	21.4	77.9	2.0

[1] Daily minimum temperature (Tn)

[2] Daily maximum temperature (Tx)

[3] Daily minimum relative humidity (RHn)

[4] Daily maximum relative humidity (RHx)

Table 7. Weather data collected in seven maize-growing districts in South Africa during February and March of the 2013 and 2014 production seasons.

Crop	Province	District	Year	Month	Average Tn (°C)[1]	Average Tx (°C)[2]	Average RHn (%)[3]	Average RHx (%)[4]	Average Rain (mm)
Maize	Northern Cape	Douglas	2013	February	12.6	36.0	14.4	90.4	0.4
				March	12.2	34.0	19.3	90.7	0.1
		Prieska	2013	February	14.4	36.0	10.5	74.2	0.1
				March	14.0	33.3	17.5	75.7	3.3
		Jacobsdal	2013	February	16.5	34.5	14.8	92.8	0.1
				March	16.0	37.6	20.6	93.1	0.1
	KwaZulu-Natal	Bergville	2013	February	15.2	30.9	38.0	97.2	5.5
				March	13.5	28.8	38.4	97.5	2.3
		Winterton	2013	February	14.6	31.1	33.9	92.6	3.9
				March	12.6	28.9	36.0	93.5	1.5
	Limpopo	Groblersdal	2014	February	17.8	33.3	33.7	95.9	2.2
				March	17.6	29.2	52.2	99.6	6.4
		Koedoeskop	2014	February	18.6	31.5	33.8	89.1	4.0
				March	17.9	27.4	53.0	93.3	4.9

[1] Daily minimum temperature (Tn)

[2] Daily maximum temperature (Tx)

[3] Daily minimum relative humidity (RHn)

[4] Daily maximum relative humidity (RHx)

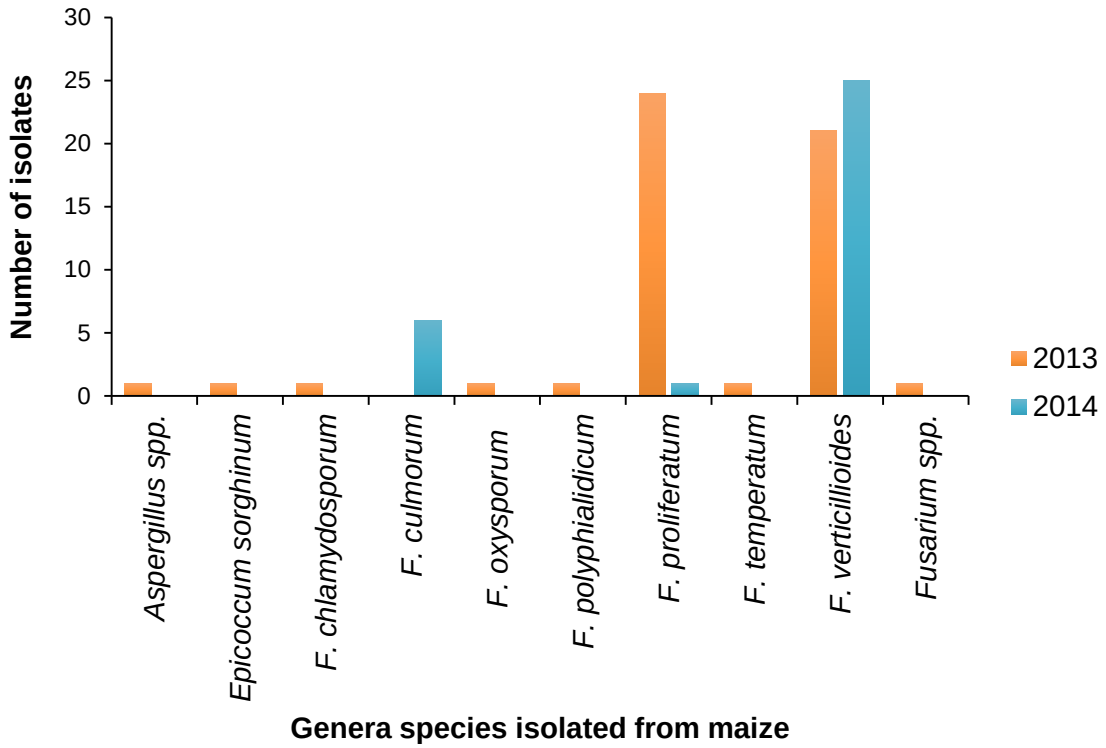

Figure 1. The number of non-*Fusarium graminearum* species complex isolates obtained from maize during 2013 and 2014 in South Africa.

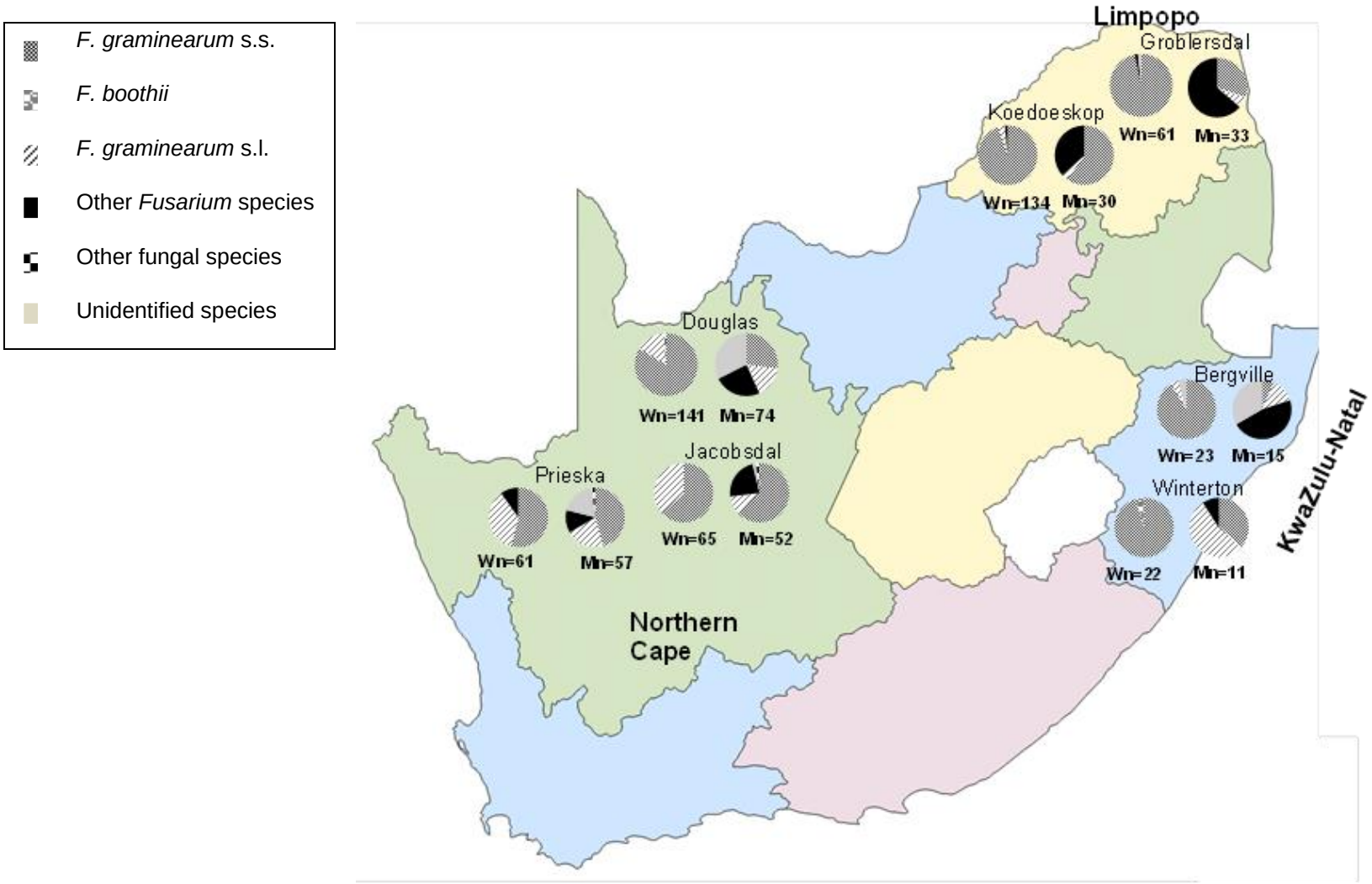

Figure 2: Geographic distribution of the number of isolates and *Fusarium graminearum species* complex species distibution from wheat (Wn) and maize (Mn) in South Africa.